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CLINICAL CHEMISTRY

Journal of the American Association of Clinical Chemists

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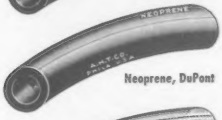
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The original (on bond paper) and first carbon of the manuscript should be submitted, triple spaced with ample margins all around. The author's last name should appear on each page. Separate pages should be used for title page (with author's name and affiliations), references, footnotes (when unavoidable), illustration legends, tables, and other inserts.

References and the design of tabular matter should follow exactly the form used in current issues of this journal. Journal abbreviations should conform to the style of *Chemical Abstracts*. The accuracy of the references and the provision of adequate bibliographic data are the responsibility of the author.

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REAGENTS and REACTIONS 3

- *Enzymatic hydrolysis for conjugated steroids*
- *Fibrinogen to explore clotting mechanisms*
- *Diagnosing electrolyte-water imbalances*
- *Fructose-free Inulin for clearance tests*

Progress in Plasma Steroid Research

Corticosteroids, conjugated as glucuronides, have for the first time been recovered quantitatively from human plasma. Administering large oral doses of "tetrahydro-E," Bongiovanni and Eberlein[1] were able to estimate the conjugated corticosteroid in the plasma of their subjects and to confirm its identity with "tetrahydro-E." For hydrolyzing the glucuronide conjugate, they used Ketodase, Warner-Chilcott's brand of beef liver β-glucuronidase because strong acid hydrolysis would have destroyed the steroid and proved inefficient in breaking the glycosidic bond. With Ketodase, however, there was full recovery of the steroid. In your research, you will find that Ketodase gives you accurate and consistent recoveries time after time. If you desire more information about steroid determinations, use the coupon below.

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1. Proc. Soc. Exper. Biol. & Med. *89*:281 (June) 1955. 2. Lorand, L.: Physiol. Rev. *34*:742, 1954. 3. J.A.M.A. *159*:771 (Oct. 22) 1955. 4. J. Clin. Investigation *34*:1520 (Oct.) 1955.

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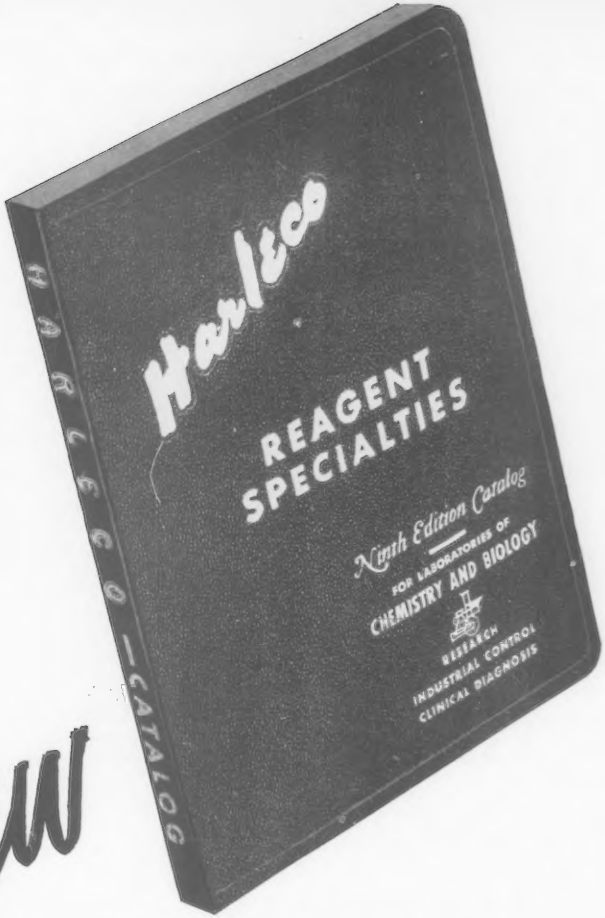

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Cholesterol in Serum and Lipoprotein Fractions

Its Measurement and Stability

Joseph T. Anderson and Ancel Keys

With the collaboration of Flaminio Fidanza,* Margaret Haney Keys, B. Bronte-Stewart,† Paul Kupcs, and Laura Werner

THE POTENTIAL IMPORTANCE of the β-lipoproteins as contributors of the cholesterol in atherogenesis suggests that extensive use of measurements of the β-lipoprotein cholesterol (BLPC), as well as of the total cholesterol in serum is desirable. For both total and BLPC, researches involving their estimation in connection with the atherogenesis problem will commonly require statistical evaluation of "before and after" values or of comparisons between population groups, two situations demanding detailed information on the reliability of the methods.

The present paper describes methods, applicable to 0.1 ml. serum samples, for total cholesterol and for its fractionation into BLPC and the α-lipoprotein cholesterol (ALPC) by paper electrophoresis and by cold ethanol precipitation. Data are given also on the storage of samples for cholesterol measurement, on the reproducibility of the several cholesterol measurements, and on spontaneous intraindividual variations of serum cholesterol concentration.

It is shown that the BLPC separated by paper electrophoresis is equivalent to that from cold ethanol fractionation and that the total cholesterol in serum and in the electrophoretically separated fractions is stable at

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Part of the costs of these studies were borne by grants from the National Dairy Council, Chicago, Ill., and the U. S. Public Health Service (grant recommended by the Cardiovascular Study Section).

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room temperature for months when it is air dried on filter paper. Finally, it is shown that the analytical errors with the present methods are smaller than the spontaneous intraindividual variability of human serum.

MATERIALS

Paper-electrophoresis apparatus. The apparatus is patterned after that built by Swahn (1, 2). A rectangular moist chamber of acrylate plastic (Plexiglas or Lucite) 230 mm. long by 210 mm. wide supports four paper strips horizontally on plastic points (at intervals of 1 to 2 cm.), so as to be midway in the vertical chamber depth of 12 to 15 mm. The ends of the strips of filter paper (Whatman No. 1), 45 x 350 mm., are bent straight down to dip into buffer solution about 45 mm. below the bed of points. A thin sponge rubber gasket assures a tight closure of the flat top plate on the edges of the side and end walls of the moist chamber.

Michaelis' veronal buffer. Five L. contain 29.428 Gm. of barbital sodium, 19.428 Gm. of sodium acetate ($NaC_2H_3O_2 \cdot 3H_2O$) and about 15 ml. of normal HCl to make *p*H 8.6.

Sudan Black B solution (Swahn) (2). One Gm. of Sudan Black B is added to a mixture of 600 ml. absolute alcohol and 400 ml. water. The mixture is stirred constantly while it is heated to boiling and boiled 10 minutes. It is cooled and filtered through a large filter of Whatman No. 1 paper. If black specks appear on the dyed papers the solution is refiltered.

Aqueous KOH, 33%. Ten Gm. of KOH plus 20 Gm. of water.

Alcoholic KOH, 2%. Six ml. 33% aqueous KOH diluted to 100 ml. with absolute ethanol.

Standard cholesterol solutions. Dissolve 60.0 mg. of pure dry cholesterol in absolute alcohol and dilute to 500.0 ml. in a volumetric flask. A 2.0 ml. portion of this contains 240 μg. of cholesterol. When used as described it corresponds to a serum containing 240 mg. per 100 ml. Portions of this solution are accurately diluted twice and six times to give 120 μg. and 40 μg. standards, respectively.

Liebermann-Burchard reagent. Place 90 ml. of acetic anhydride in a glass-stoppered Erlenmeyer flask. Cool in ice to 8° or colder. Keep cold while adding, dropwise, 4.5 ml. of concentrated H_2SO_4 with constant stirring. Let stand 5–10 minutes in the ice bath, add 45 ml. of glacial acetic acid and bring to 25° before use. Use within an hour.

ACD solution. One L. contains 22.0 Gm. of trisodium citrate dihydrate, 8 Gm. of citric acid monohydrate, and 22.0 Gm. of dextrose.

Sodium acetate buffer. Two hundred ml. of 4M sodium acetate and 400 ml. of 10M acetic acid are diluted with water to make 1 L. This buffer when diluted 1:80 with distilled water should have a *p*H 4.00 ± 0.02 at 25°.

Alcohol-buffer. Add 2.4 ml. of the above acetate buffer to 250 ml. of 95% ethanol and dilute with water to 1 L. This should be prepared fresh daily.

CALCULATIONS

In the analysis of the results, both of duplicate measurements with the same method and of the application of different methods to the same serum, we have used the following statistics

Mean Difference

$\bar{\Delta} = \Sigma\Delta/N$, where Δ is the difference (having regard to sign) between first and second measurements and N is the number of pairs of measurements.

Standard Deviation of the Differences (S.D. Δ)

$$(S.\ D.\ \Delta)^2 = \frac{N\Sigma\Delta^2 - (\Sigma\Delta)^2}{N\ (N - 1)}$$

Note that where there is no difference between the means of the first and second measurements this value is almost equal to $S.\ E.\ M.$ multiplied by the square root of 2 ($= 1.414$), where $S.\ E.\ M.$ is standard error of measurement.

Standard Error of Measurement (S.E.M.)

$$(S.\ E.\ M.)^2 = \Sigma\Delta^2/2N$$

Standard Error of the Mean Difference

$$S.\ E.\ \bar{\Delta} = S.\ D.\ \Delta/\sqrt{N}.$$

Percentage Mean Difference

These values are obtained by multiplying the above values by 100 and dividing by the grand mean of the measurements in the series. For example:

$$\bar{\Delta}\% = 100\bar{\Delta}/\tfrac{1}{2}(\bar{X}_1 + \bar{X}_2).$$

TOTAL CHOLESTEROL MEASUREMENT

In the present methods the final estimation of cholesterol is essentially the same for the BLPC as for the total cholesterol in 0.1 ml. serum, so it is useful to consider this first. This is an adaptation of the method of Abell *et al.* (3), in which cholesterol esters are hydrolyzed with alcoholic KOH, total cholesterol is extracted from the alcoholic solution by pe-

troleum ether (B.P. 60–80°), and, after evaporating the solvent, the Liebermann-Burchard reaction is utilized. Our version, which we call the K5 method, has been applied to thousands of samples in five different laboratories and the experience with this method and some details of the procedure are briefly indicated below.

Procedure

Two ml. of fresh alcoholic KOH are added to the measured sample (0.1 ml. of serum) and to standards and reagent blanks, in glass-stoppered test tubes. Hydrolysis is complete within 90 minutes at 37°. This system is stable, so the stoppered tubes may be left for at least a week in an incubator or for several weeks at room temperature before proceeding further. A 2-ml. portion of water is then added and the mix is extracted by shaking briefly (60 seconds) with 4-ml. portions of redistilled petroleum ether and transferring the supernatant extracts with a capillary (Pasteur) pipet directly to the Evelyn colorimeter tube. Two extractions, with a final rinse of the pipet, extract 98 to 99 per cent of the cholesterol. With three extractions only a very rough and hasty technic in transfer is needed for complete recovery.

After adding 1 drop of 25% acetic acid in petroleum ether to each tube, the solvent is evaporated by a stream of warm air directed down into the tube. When the tube appears dry when held to the light, the color development can be started, or this may be delayed for weeks, if need be, because the dry residue in the tube is stable.

Colorimeter Readings

In the development of color with the Liebermann-Burchard reagent we find it essential, especially when working in the field or when using varied batches of chemicals, to make serial readings on each tube to assure recording the actual maximum with a 620 mμ filter. At 20–25° this usually means starting readings at 20 minutes and repeating at 5-minute intervals until the maximum is known. With the Evelyn photoelectric colorimeter, readings at 15-second intervals are convenient, so a batch of 20 tubes at a time is suitable.

REPRODUCIBILITY

The reproducibility of this K5 method has been extensively studied and it has been repeatedly checked with digitonin precipitation methods and with the simpler Bloor extract procedure in which hydrolysis is omitted and the mixed free and ester cholesterol is extracted with Bloor's

Table 1. Reproducibility, in Four Laboratories, of the K5 Method for Total Cholesterol in 0.1 Ml. of Serum from Healthy Men

There were 100 pairs of duplicate analyses in each series except in Minneapolis where the number was 118.

	Minneapolis	*Naples*	*Cape Town*	*Bologna*
S.E.M.[a] (mg./100 ml.)	±3.52	±2.96	±4.52	±3.57
S.E.M.%[b]	±1.42	±1.64	±2.44	±1.79
r[c]	0.979	0.992	0.979	0.978

All series used 100 pairs of duplicate analyses except Minneapolis, using 118.

[a] Standard error of measurement.

[b] Percentage of the mean of the series.

[c] Coefficient of correlation.

alcohol-ether mixture. Data on simple replication in four laboratories are summarized in Table 1.

It will be observed that the reproducibility of results with the K5 method is such that, in general, two out of three replications will depart less than ±2 per cent from the first measurement. The poorest reproducibility we have seen was in the Cape Town series summarized in Table 1, where the work was done under great pressure of time with an unstable photoelectric colorimeter. The high coefficient of correlation in the Cape Town series reflects the wide range of cholesterol (90 to 400 mg./100 ml.) in the series.

As shown in Table 2, the K5 method tends to produce values very slightly higher than from the digitonin methods of Sperry and Webb (4) and of Foldes and Wilson (5). This might be explained on the theory that the K5 method gives a slight overestimate because the Liebermann-Burchard reaction as used in K5 is not completely specific. It seems equally plausible that the digitonin methods give slight underestimates

Table 2. Comparison of Results from Present (K5) Method with Those from the Digitonin and the Bloor Extract Methods in Two Laboratories

	K5–Digitonin[a] *(Minneapolis and Naples)*	*K5–Bloor*		*Bloor–Digitonin*[b]
		Minneapolis	*Naples*	
N	20	134	27	50
$\bar{\Delta}$ (mg./100 ml.)	5.7	−44.0	−49.2	36.0
S.E. $\bar{\Delta}$ (mg/100 ml.)	±2.0	±0.88	±4.0	±8.6
$\bar{\Delta}$ %	2.3	−19.6	−30.3	15.9
S.E. $\bar{\Delta}$ %	±0.5	±0.39	±2.5	±3.8

[a] Comparison made with method of Foldes and Wilson (5).

[b] Comparison made with method of Sperry and Webb (4).

because the digitonide is not absolutely insoluble in the solvents used. For most purposes the difference is negligible but in any case the digitonin result can be computed at 0.97 times the K5 result or, conversely, K5 = 1.03 × digitonin. In the series of 20 samples covering the range 100 to 292 mg./100 ml., in Table 2, the coefficient of correlation between the means of duplicates from the two methods was $r = 0.9918$, the mean value by K5 being 211.3 and by digitonin being 205.6 mg./100 ml.

The comparison of Bloor extract values with those from the Sperry and Webb digitonin method gives, as expected, a difference similar to that found when the K5 method is compared with the Bloor extract. On the average, the Bloor extract value is 15.9 per cent too high but the discrepancy is reasonably consistent and the coefficient of correlation between Bloor extract and digitonin values in 50 samples was $r = 0.87$.

The discrepancy between the K5 and the Bloor extract values is far larger and more serious. Primarily this stems from the fact that the color intensity resulting from the Liebermann-Burchard reaction acting on cholesterol esters is higher than that with free cholesterol, so the Bloor values, which are obtained from a mixture of free and ester cholesterol but computed as though all were in the free form, are erroneously high. With storage the proportion of free to ester cholesterol may be changed by the action of esterases in the serum (6). The data in Table 1 refer to serum started through analysis within 1 to 3 hours of blood drawing. For this condition the finding in Minneapolis on 134 bloods, each analyzed in duplicate by the K5 and by the Bloor extract method, is that the K5 result can be predicted by multiplying the Bloor extract value by the factor of 0.836, the standard error of estimate being ±10.24 mg./100 ml.

PAPER ELECTROPHORESIS PROCEDURE

A pencil line is drawn across each paper strip at a point which will be about 3 cm. from the cathode end of the bed of the moist chamber. The paper is dipped in buffer, blotted until the gloss disappears, placed in the moist chamber bending down the ends to dip in buffer in the electrode vessels, and covered for an hour or more before applying 0.1 ml. of serum in a single point at the center of the pencil line. Good results are obtained also if the serum is applied to the dry paper by spreading it uniformly along the pencil line, keeping 5 mm. away from each edge of the paper and immediately adding the buffer dropwise to the remainder of the paper.

The moist chamber is covered and a D.C. potential of 185 volts is applied for 15 hours. The strips are dried by hanging them by one end in

room air or by warming on a smooth clean surface (e.g., a photographer's print drier). As will be seen, in this dry state the paper may be stored for at least many weeks before proceeding further.

It is desirable to run, in parallel, two strips for each sample, one to be stained as a guide for cutting the other strip into α- and β-lipoprotein parts. When the dry strips are examined before staining two brown spots are usually visible, one having migrated with the albumin about two thirds of the length of the strip, while the other has migrated only about half as far and is in the globulin region. Further, when the strip is examined under ultraviolet light, a large fluorescent spot appears, the albumin brown spot being within this. The positions of these spots, which may be outlined with pencil, are useful guides for cutting the paper so as to separate the α- and β-lipoproteins.

The uncut strip to be dyed is immersed in Sudan Black B solution for 10–60 minutes and then is briefly washed in ethanol-water (1 to 1) and is dried. If there is any doubt that this shows where to cut the undyed strip, the pencil marks made on the two strips as noted above may be compared.

Extraction of the Electrophoresis Paper Strips

Each portion of paper is rolled and placed in a glass-stoppered test tube of 13–18 ml. capacity. Two milliliters of 2% alcoholic KOH are added, and the tube is inclined so as to wet the paper and is incubated at 37 to 40° for at least 90 minutes. A 4-ml. portion of petroleum ether is added, followed by a 6-ml. portion of ethanol-water (1 to 2). The tubes are shaken for 60 seconds, allowed to stand briefly and the petroleum ether layer is transferred to an Evelyn colorimeter test tube by a Pasteur pipet with a 2-ml. rubber bulb. After use the pipet is conveniently washed with petroleum ether from a polyethylene wash bottle, the washings being collected in the colorimeter tube. The petroleum ether extraction is repeated twice more, using 4-ml. portions each time. One drop of 25% glacial acetic acid in petroleum ether is added to each tube and the solvent is evaporated as in the total cholesterol analysis.

Reagent blank tubes and standard tubes are prepared by similar procedures. Reagent blanks are made with alcoholic KOH, incubated, and extracted in the same way as the serum samples. Filter paper and buffer salts are not added since they were found to contribute no color. Standards are set up by measuring 2-ml. portions of alcoholic cholesterol standard solutions into glass-stoppered tubes. A dry strip of buffer-treated paper, 120 mm. long, is placed in each of the tubes for "beta-

standards" which should contain amounts of cholesterol in the range expected for the β-lipoprotein samples. Two "alpha standard" tubes containing 40 μg. of cholesterol are given 50-mm. portions of similar paper. To each standard tube is added 0.12 ml. of 33% aqueous KOH. The tubes are incubated and thrice extracted with petroleum ether in the same way as the other tubes. The rest of the analysis is done as in the ordinary cholesterol measurement.

COLD ETHANOL FRACTIONATION PROCEDURE

This is a small-scale adaptation of the first step of Cohn's Method X for blood protein fractionation (7, 8, 9). Alpha-lipoprotein cholesterol appears in the supernatant fluid after the precipitation while the β-lipoprotein cholesterol appears in the precipitate.

To 0.1 ml. of serum in a glass-stoppered round-bottomed centrifuge tube is added 0.03 ml. of ACD solution and the whole is cooled to −5° with constant stirring by a plastic-enclosed magnetic particle. Then, slowly, 0.52 ml. of fresh alcohol-buffer is added while stirring continues. Centrifuging for 30 minutes at −5° completes the sedimentation and the supernatant can be decanted and drained off (at −5 to −10°).

The residue is dissolved in 2 ml. of alcoholic KOH, incubated at 37° for 90 minutes, treated with 2 ml. of water and then is extracted twice with 4-ml. portions of petroleum ether. The cholesterol determination is completed as described above for total serum cholesterol by the K5 method.

RESULTS FROM DUPLICATE FRACTIONATIONS

Replication studies on the β-lipoprotein cholesterol (BLPC), both from paper electrophoresis and from cold ethanol separation, are summarized in Table 3. In our experience the agreement between duplicates tends to be better in the cold ethanol method (S.E.M. = ±4.3 vs. ±5.8, the

Table 3. Replication Studies on the Estimation of Cholesterol in β-Lipoprotein (BLPC) as Separated by Paper Electrophoresis and by Cold Ethanol

ALPC from cold ethanol (Sum) with the total cholesterol measured by the K5 method.

Comparison	*N*	$\bar{\Delta}$	$\bar{\Delta}$%	*S.D.* Δ	*S.D.* Δ %
Paper–paper	36	..	..	±8.1 mg.	±4.0
Ethanol–ethanol	169	..	..	±6.1 "	±3.0
Paper–ethanol	128	1.0 mg.	0.5	±13.5 "	±6.8
Sum[a]–K5[b]	128	5.3 mg.	2.2	±12.2 "	±5.0

[a] Sum of BLPC and ALPC from cold ethanol.
[b] Total cholesterol measured by K5 method.

difference being slight but highly significant in large series). But with both methods at least two thirds of second analyses fall within ±4 per cent of the BLPC value obtained in the first analysis.

On the average, the estimation of BLPC from paper electrophoresis agrees reasonably well with that from cold ethanol, as shown in Table 3. The correlation coefficient between results with the two methods is $r = 0.953$.

The comparison of the sum of the ALPC and BLPC with the total cholesterol measured directly with the K5 method indicates a small and fairly consistent discrepancy, such that ALPC plus BLPC gives an average overestimate of 2.2 per cent of the K5 result, as shown in Table 3. It should be noted that the standard deviation of the difference between the sum of the paper strips and the direct (K5) total cholesterol measurement includes the effects of three measurement errors. The standard error of measurement of α-lipoprotein cholesterol (ALPC) is somewhat smaller than that for BLPC and the standard error of measurement of the sum of ALPC and BLPC was ±6.70.

The percentage of total cholesterol in the β-lipoprotein fraction was computed by both the paper electrophoresis and the cold ethanol methods in 128 serum samples. The mean values were 80.9 and 82.4 per cent, respectively, and the S.D. Δ between methods was ±4.0 per cent. In 36 such duplicate estimates with the paper electrophoresis method the range was 63 to 93 and the standard error of measurement ±1.1 per cent of total cholesterol. Using the cold ethanol fractionation method for BLPC and the K5 method for total serum cholesterol the percentage of total cholesterol in the beta fraction was computed in duplicate for 164 comparable cases. The range was 63 to 97 and the standard error of measurement ±2.1 per cent.

STORAGE STUDIES

It is well known that it is hazardous to store nonsterile blood for more than a day or two in an ordinary refrigerator if cholesterol is to be measured. But repeated studies in this laboratory have shown that the concentration of total cholesterol in nonsterile human blood serum stored at −20° is stable for at least 5 years, though sampling presents difficulties because the thawed system is a mixture of phases. These results refer to cholesterol as measured by a method involving hydrolysis of the esters and subsequent application of the Liebermann-Burchard reagent; without this step variable results are obtained because of spontaneous hydrolysis and esterification in the stored serum (6).

Besides deep-freeze storage, we have found that total serum cholesterol

is well maintained for many months at room temperature in lyophilized (dry) serum. This is a convenient way to maintain a reference standard for checking analytical methods. It is not even necessary to use proper freeze-drying to get a stable product. Small quantities of serum (up to 1 ml.) may be dried without cooling in a wide-mouthed vial placed in a vacuum line close to an open supply of anhydrous magnesium perchlorate or phosphorous pentoxide for 1 hour. For example, an average of 234.5 mg./100 ml. of total cholesterol was obtained from 8 analyses on 2 fresh human serum samples. Fourteen analyses on the same serums vacuum-dried in this simple fashion and then stored for 30 days at 37° gave an average of 235.3 mg./100 ml.

Air-Dried Samples of Serum

These results led to further attempts at simplification and it was found that the total cholesterol in small samples (0.1 ml.) of serum may be preserved for at least several months at temperatures of 25–30° merely by air drying on filter paper. The serum is measured out so as to cover the maximum area (2 to 3 cm. in diameter) on Whatman No. 1 paper which is then hung up in the air for a few hours to dry.

The subsequent analysis, of course, requires extraction of the cholesterol from the dried sample of filter paper but this can be combined with the hydrolysis step so there is no great addition to the labor of analysis. The procedure described above for the analysis of paper electrophoresis strips is used, but we find that more satisfactory extraction results if the temperature is maintained at 70° for the combined hydrolysis-extraction step.

A series of 126 samples of serum drawn in Cape Town, covering a range of from 82 to 424 mg. of total cholesterol, were studied in this way. The mean for the fresh serums analyzed, each in duplicate, at Cape Town was 196.1 ± S.E. = 6.3 and for the paper-dried replicates done at Minneapolis, in duplicate, from 3 to 6 weeks later, the mean was 197.7 ± 5.5, the coefficient of correlation between the two analyses being $r = 0.986$. The average difference, without regard to sign, between the analyses done on the fresh and on the dried serums was 8.0 mg./100 ml., and the standard deviation of the differences between results on fresh and on dried serum was ±9.8 mg./100 ml.

A second test, under somewhat more favorable conditions for the measurements of cholesterol in the fresh serum, was made in Italy. Blood samples were taken from 112 clinically healthy men in Cagliari, Sardinia. After separation of the serum, 0.1-ml. portions were dried on paper and the remainder placed in screw-capped vials and stored in cold thermos

jugs at 0° until analyzed in Naples a few days later. The dried samples on paper were stacked up, wrapped in Saran Wrap and were mailed to Minneapolis. The mean from the fresh material at Naples was insignificantly (0.2 mg./100 ml.) lower than the mean from the papers analyzed at Minneapolis, the average difference between the two methods, without regard to sign, being 5.4 mg./100 ml. The standard deviation of these differences was ±6.9 mg./100 ml.

The paper strips after paper electrophoresis may be dried similarly and analyzed at leisure many weeks later. No attention need be paid to the temperature of storage. Staining the strips to identify the lipoprotein distribution may be done at any stage between electrophoresis and final extraction and completion of the analysis.

DISCUSSION

Metabolic Influences

The bloods used in the foregoing analysis of the results with the various cholesterol methods were all from clinically healthy men. These methods have also been applied to blood from hospital patients with a variety of diseases. The agreement between duplicates analyzed for total cholesterol by the present K5 method in several series of patients was similar to that found for healthy subjects. However, somewhat greater variability between replicates in paper and cold ethanol fractionation was found among patients with disordered cholesterol and/or lipid metabolism in general (cirrhosis, nephrosis, diabetes). In a series of 11 such patients the sum of ALPC plus BLPC on paper averaged 7.5 mg./100 ml. higher than the direct measurement of the total cholesterol, the standard deviation of the differences being ±12.9 mg./100 ml. or ±5.0 per cent of the mean total cholesterol. In this same series of 11 patients the BLPC estimation from paper electrophoresis averaged 9.6 mg./100 ml. higher than the BLPC separated by cold ethanol and the standard deviation of the differences was ±25.6 mg./100 ml. Even when allowance is made for the average difference between the BLPC results with the two methods, the standard deviation of the difference was ±10.8 per cent of the grand mean as compared with ±6.9 per cent for "normal" men and this is highly significant statistically. Apparently in these very abnormal bloods there may be unidentified factors which reduce the reproducibility of the BLPC results.

Intraindividual Variations

The practical effect of the method error in any analytical method should be judged after due consideration of the limitations imposed by

the presence of other uncontrolled or uncontrollable variations present in the practical situations in which the method is applied. In the case of cholesterol in human serum, spontaneous, intraindividual variation is a factor to be considered whenever the problem concerns "before and after" measurements.

In order to compare the analytical error of the present methods with these true intraindividual variations, we adopted the following general scheme. With suitably selected subjects, blood samples were drawn on two occasions, A and B, and analyses 1 and 2 were performed in duplicate on each sample. The differences between analyses A1 and A2 and between B1 and B2 give a measure of the method reproducibility, while the differences between samples A1 and B1 and between A2 and B2 include both method error and the intraindividual variability. The results of this procedure in three series of men, all studied in the basal fasting state, are given in Table 4.

Variability in the method itself accounts for no more than a small fraction of the variation between occasions in any of the series. The healthy young soldiers, living under highly constant controlled conditions in the laboratory, are the most stable group but even in them the standard deviation between occasions is four times that between replicates of the same serum. The intraindividual variability of the obese young men, and particularly of the coronary patients, is greater. In the face of such spontaneous variation of patients on what is supposed to be a constant

Table 4. METHOD, OCCASION, AND TRUE INTRAINDIVIDUAL VARIABILITY OF TOTAL CHOLESTEROL AND β-LIPOPROTEIN CHOLESTEROL CONCENTRATIONS

Series[a]	Item	N[b]	Grand mean	Variance of differences			Standard deviation of differences		
				Method	*Occasion*	*"Indiv."*	*Method*	*Occasion*	*"Indiv."*
A	Total cholesterol	22	209.5[c]	19.97	400.6	380.6	4.47	20.02	19.51
B	β-Lipoprotein cholesterol	13	169.8	11.52	504.8	493.2	3.40	22.47	22.21
B	Total cholesterol	13	214.3	21.16	558.9	537.8	4.60	23.64	23.19
C	β-Lipoprotein cholesterol	24	242.7	67.71	1447.8	1380.1	8.22	38.05	37.15
C	Total cholesterol	24	273.4	23.46	900.3	876.8	4.84	30.00	29.61

[a] Series A = healthy soldiers studied on a constant regimen in the laboratory on 2 occasions one week apart. Series B = healthy obese young men studied on 2 occasions 4 days apart during a control period with diet *ad lib*. Series C = ambulant middle-aged men with coronary heart disease studied, while supposedly on a constant regimen of diet and exercise, on 2 occasions 3 months apart.

[b] N = Number of men (occasions = 2N, analyses = 4N).

[c] All values in mg. of cholesterol per 100 ml.

regimen it is obvious that these are serious limitations to the demonstration of the effects of therapy with limited numbers of patients. It should be recognized that the values for intraindividual variation given in Table 4 will not be reduced by taking the mean values of duplicate analyses of the same bloods. For total cholesterol, a direct trial of this calculation, using the means of duplicate analyses on each occasion in Series C of Table 4, gave, as predicted, variance = 888.8 for the gross individual difference between occasions.

Testing for Effect of Drugs

Such data on replicate variances ascribable to method error and to intraindividual variability are, of course, basic guides for planning research projects. Suppose it is desired to test the effect, if any, of a drug that is reputed to reduce the serum cholesterol and, accordingly, analyses will be made on serums from patients before and after getting the drug. Assume that that method and intraindividual variances, apart from any systematic effect of the drug, will be similar in the control and drug periods. What then, can be predicted about the number of patients required to "prove", at $p = 0.01$, an effect of the drug amounting to an average fall of 20 mg./100 ml. in total cholesterol? Alternatively, what is the least average fall to prove the point with a given number of patients?

With single analyses on one control and one experimental occasion the values for $p = 0.01$ are: $t = 3.25$, 2.86, 2.76 for 10, 20, or 30 patients, respectively. From Table 4 it is calculated that the standard errors to be expected for the mean "occasion" difference with these numbers of patients will be ± 9.49, ± 6.71, and ± 5.48 mg./100 ml., respectively; for 20 mg./100 ml. mean change the corresponding t values are 2.10, 2.98, and 3.65. Hence 20 would be a good estimate of the fewest patients to be studied.

Alternatively, with 20 patients, it is easy to estimate the least mean change that would be highly significant $\bar{\Delta}/6.71 = 2.86 = t$, or $\bar{\Delta} = 19.2$ mg./100 ml. (for $p = 0.01$).

Eliminating the method error completely by making a large number of replicate analyses on the same bloods would not significantly change the foregoing indications. With perfect analyses, the standard error of the mean difference between occasions with 20 coronary subjects would not be less than $29.61/\sqrt{20} = \pm 6.62$ mg. total cholesterol per 100 ml., so to reach $p = 0.01$, $\bar{\Delta}$ would have to be 18.9 mg./100 ml.

Obviously, it is more important to reduce the "occasion" variance Other things being equal, with single samples on each of two control and

two "drug" periods, the standard error of the mean difference between occasions would be reduced by almost one third compared with that from single occasions. With 20 coronary patients it should approach ±4.74 mg./100 ml. for total cholesterol with samples on each of two occasions.

Addenda

Since the foregoing was written, a paper by Oliver and Boyd (10) has been published which adds further weight to the argument for the importance of the BLPC. Their cholesterol method does not involve hydrolysis of the esters and is not suitable for use in photoelectric colorimeters requiring much more than 1 ml. of solution for final color reading.

While this paper was being processed for publication, results were obtained of a check on the present method of preserving serum dried on filter paper and subsequent cholesterol analysis by our K5 method. Dr. George S. Boyd, Department of Biochemistry, University of Edinburgh, kindly pipetted and dried on filter paper duplicate samples of 20 serums and sent these to Minneapolis for analysis without disclosing his results obtained by the Sperry-Schoenheimer procedure applied to the fresh serum. The Minneapolis results averaged 4.6 per cent lower than the Edinburgh results, the standard error of measurement being ±1.6 per cent of the mean. Most of the small average discrepancy was contributed by two serums for which the Minneapolis values were 344 and 278 mg./100 ml. while the respective Edinburgh values were 302 and 225 mg./100 ml. These findings conform with our experience that the digitonin method generally gives slightly lower values, some of which, at least, probably represent incomplete precipitation or loss of digitonide.

SUMMARY

1. Methods are described for the separation, by paper electrophoresis and by cold ethanol, of α- and β-lipoproteins in 0.1 ml. of serum, with subsequent analysis of cholesterol in the separated portions.

2. It is shown that both methods of separation yield separated fractions containing substantially the same amounts of cholesterol.

3. Detailed data are given on the errors of measurement for total cholesterol and for cholesterol in the separated lipoprotein fractions.

4. Studies are reported on the stability of cholesterol in stored serum and on paper electrophoresis strips. It is shown that simple drying on filter paper causes no change in cholesterol content and yields a product that is stable for many weeks at ordinary room temperature.

5. The sources of variability in human serum cholesterol values are

examined and it is shown that spontaneous intraindividual variability is a much greater source of error than the errors of measurement with these methods.

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Azorubin in Aqueous and Methanolic Solutions

Spectrophotometric Studies

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DEVELOPMENTAL STUDIES

The original quantitative procedure for bilirubin of Van den Bergh (1) was based upon comparison of the color of an ether extract, first with ferric thiocyanate solutions and later with cobalt sulfate standards. This inaccuracy of standardization has been eliminated by the availability of purified bilirubin in both solid form and in comparatively stable solutions, and by the adaptation of the procedure to spectrophotometers and photoelectric colorimeters, both of which isolate narrow bands of light as compared to the entire spectrum used with visual colorimeters.

The second problem was that of precipitation of the proteins by the addition of ethanol in the determination of total bilirubin, accompanied by subsequent loss of some bilirubin by adsorption, and by incomplete dissociation from the assumed protein-bilirubin complex. The solution to this problem was worked out by Malloy and Evelyn (2), who found that the serum proteins did not precipitate in 50% methanol provided the final protein concentration was below a certain level. At this concentration of methanol, full color development of the azorubin takes place. The final dilution in the original method was about 1 to 40 or 50, with a protein concentration of 300–400 mg. per 100 ml. Observations during the present study indicate that protein precipitation in methanol does not occur under the proposed test conditions, if the serum dilution is 1 to 8 or more—that is, a protein concentration of not more than 1%. Above this protein concentration precipitation almost invariably occurs.

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The division of serum bilirubin into prompt, biphasic, and delayed reactions has been shown by Gray and Whidborne (3) to depend in large part upon the total concentration of direct bilirubin. They pointed out that when the serum direct bilirubin concentration was greater than 10 mg. per 100 ml., a "prompt" reaction always occurred. When the concentration was less than 5 mg./100 ml., the formation of azorubin is so slow that the reaction is called "delayed." With concentrations between 5 and 10 mg./100 ml., the "biphasic" reaction is most commonly observed. These concentration ranges were determined with rather large dilutions of serum. It is quite likely that with a dilution of 1 to 8 biphasic reactions will be observed in concentrations as low as 2 mg./100 ml.

Several workers (4, 5, 6) have pointed out that Watson's (7) 1-minute direct bilirubin is merely an arbitrary point on a time rate-color development curve. It takes about 7 minutes to develop 95% of the color of azorubin, regardless of the concentration of direct bilirubin. Therefore, since the shape of the curve is determined by the total direct bilirubin concentration, and since the 1-minute concentration is merely a point on this curve, the 1-minute bilirubin has no more clinical significance than the total direct bilirubin, upon which it depends.

It remained, therefore, to determine whether "direct" and "indirect" bilirubin actually exist in serum, or whether they are artefactual.

EXPERIMENTAL AND RESULTS

The apparatus used was the Coleman Junior Spectrophotometer, Model 6A, with 12 × 75 mm. cuvets.

The reagents were those of Malloy and Evelyn (2). The bilirubin stock standard solution (Hartmann-Leddon Company) contained 10 mg. of bilirubin in 100 ml. of chloroform. This solution was kept in the refrigerator when not in use. The factor determined from this standard, which has been used for calibration of the spectrophotometer for routine bilirubin determinations in this laboratory, has not changed in more than 3 years, using the same bottle of stock standard.

Methanol Dilution

Dilute standard solutions of bilirubin were prepared by diluting 1, 2, 3, 4, and 5 ml. of the chloroform solution to 10 ml. with methanol. These solutions contained 1, 2, 3, 4 and 5 mg. per 100 ml., respectively. A reagent blank was prepared using chloroform instead of the stock standard.

Table 1. Absorbence of Azorubin with Methanol Dilution

Dilution factor	*MeOH conc.* (%)	*Concentration of standard* (mg./100 ml.)					
		Chloroform blank	*1*	*2*	*3*	*4*	*5*
1/4	75	0	0.220	0.435	0.635	0.865	1.080
1/8	87.5	0	0.110	0.220	0.330	0.435	0.540
1/12	92	0	0.075	0.150	0.220	0.295	0.360
1/16	94	0	0.055	0.110	0.165	0.220	0.270

Procedure

Half-milliliter aliquots of each standard were placed in a series of 12 × 75 mm. cuvettes. To each tube was added 1 ml. of methanol. After mixing by tapping the tubes 0.5 ml. of diazo working reagent (2) was added. Thus each test solution contained 75% methanol. After the solutions were mixed and allowed to stand for 15 minutes at room temperature, the absorbences were read at 540 mμ, with the absorbence of the blank tube set at 0. Two milliliters of methanol were then added to each tube, the tubes mixed by inversion, and the absorbences read again. Two milliliters of each solution were transferred to a second series of cuvets, 1 ml. of methanol added, the tubes mixed, and the absorbences read as before. The absorbences were again determined after adding another 1 ml. of methanol. All tubes were run in triplicate. The results are shown in Table 1.

Water Dilution

Another set of solutions was prepared exactly as above. After the solutions were allowed to stand 15 minutes, the absorbences were read. Distilled water was added to each tube in 2-ml. aliquots, the solutions mixed, and the absorbences read again. Two milliliters of each solution were then added to 2 ml. of distilled water in another series of cuvets, the tubes mixed, and the absorbences read. One milliliter of each solution was then transferred to 2 ml. of distilled water, the tubes mixed, and the absorbences read again. All tests were run in triplicate. The results are shown in Table 2.

Methanol-Water Dilution

Similar experiments were carried out with serum specimens from which 0.5 ml. of serum was added to 1.5 ml. of diazo working reagent and allowed to stand at room temperature for 15 minutes after mixing. The absorbence was read against a similarly prepared blank. Two

Table 2. Absorbence of Azorubin in Methanolic Solution with Water Dilution

Dilution factor	*MeOH conc. (%)*	*Concentration of standard* (mg./100 ml.)					
		Chloroform blank	*1*	*2*	*3*	*4*	*5*
1/4	75	0	0.220	0.440	0.650	0.860	1.080
1/8	37.5	0	0.105	0.210	0.320	0.425	0.530
1/16	19	0	0.050	0.100	0.150	0.200	0.250
1/48	6	0	0.015	0.030	0.045	0.060	0.075

Table 3. Absorbence of Serum Azorubin with Methanol-Water Dilution

Patient	*Absorbence*[a]		
	Direct bilirubin in H_2O	*Total bilirubin in 50% MeOH*	*After dilution to 25% MeOH*
K.V.	0.860	0.550	0.225
R.L.	0.560	0.340	0.145
F.S.	0.040	0.100	0.015
J.J.	0.780	0.460	0.200
F.L.	0.360	0.240	0.100
A.R.	0.320	0.200	0.085
L.S.	0.000	0.040	0.005
T.K.	0.500	0.280	0.130
J.R.	0.460	0.280	0.120
E.J.	0.160	0.140	0.050

[a] Factor = 10 × absorbence for direct bilirubin, 20 for total bilirubin, and 40 for last column.

ml. of methanol were then added to each tube, and the absorbences read again after mixing and standing for 15 minutes. Two milliliters of the mixture were then added to 2 ml. of distilled water, bringing the methanol concentration down to 25%. In each case, as seen in Table 3, the final absorbence was less than one half that in 50% methanol, and very close to one quarter that of the first absorbence reading in water alone.

DISCUSSION

The small amount of chloroform present in the standard solutions did not cause precipitation or cloudiness when distilled water was added to any of the test mixtures.

For the purpose of interpretation, one must assume that after 15 minutes all of the bilirubin is converted to azorubin, and that this dye is comparatively stable—that is, it does not decompose or dissociate appreciably upon dilution with methanol or distilled water. From Table

1, it is obvious that no decomposition occurs upon dilution with methanol.

In Table 1, all absorbences were proportional in each tube to the final dilution with methanol, as well as proportional to the concentration in each tube, up to an absorbence of more than 1.000. Thus, Beer's law is followed over absolute bilirubin concentrations from 0.001 to 0.25 mg. per 2 ml. of test solution.

When the original test solutions are diluted 1:12 with water, in successive steps, to a final dilution of 1 to 48, as shown in Table 2, the decrease in absorbence is not proportional to the dilution. This is observed even in a final dilution of only 1 to 8, in 37.5% methanol. However, the absorbence is proportional to the concentration for any given concentration of methanol. Theoretically, the absorbence of Tube 5, (5 mg./100 cc.) should decrease from 1.080 to 0.090 in a 1:48 dilution. The actual absorbence is 0.070, or 78 per cent of the theoretical. Thus, of 5 mg./100 cc., only 3.9 mg./100 cc. are recovered in aqueous solution. Of 4, 3, 2 and 1 mg./100 cc., only 3.4, 2.5, 1.8 and 0.8 mg./100 cc. are recovered, or 78, 85, 83, 90 and 80 per cent, respectively.

Most clinical laboratory workers find that these figures represent approximately the percentages of direct bilirubin out of the total serum bilirubin which are determined in almost every range of concentration except the low range below about 1.5 mg./100 cc. Also excluded are instances of bilirubinemia due to hemolytic causes, for it is fairly well established that in these cases we are dealing with a more or less definitely defined excess of bilirubin-globin complex, which does not diazotize completely until alcohol is added.

CONCLUSIONS

Thus, the author feels that it is reasonable to conclude that a large part, if not all, of the differences in values observed between direct and total bilirubin in serum is artefactual, and due to the difference in absorbence of azorubin in aqueous and alcoholic solutions.

Although at first glance it is difficult to reconcile this conclusion with the observed renal threshold for bilirubin, which is about 1.4 mg. of direct bilirubin per 100 ml., one soon realizes that the 1.4 mg./100 ml. may actually be 1.6 to 1.9 mg./100 ml. of a single type of bilirubin. It also becomes apparent that, whereas in obstructive and regurgitative types of jaundice only one type of bilirubin is probably present, in hemolytic jaundices the existence of two types may still be required to explain the observed Van den Bergh reactions.

SUMMARY

A study of the spectrophotometric characteristics of azorubin in varying proportions of methanol and water has been presented.

The absorbence of azorubin in water was found to vary from 73 to 90 per cent of that in methanol, over a concentration range of 0.001 to 0.025 mg. per 2 ml. of test solution.

The absorbence of azorubin was found to be linear over the same range of concentration, and over the range from 0.1 to 10 mg. per 100 ml. of serum, using a final test dilution of 1 to 8.

The strong probability of the artefactual nature of "direct" and "total" serum bilirubin values has been demonstrated.

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Metabolites of Salicylic Acid in Urine

Quantitative Determination

S. L. Tompsett

ALTHOUGH SALICYLATES are used on an extensive scale as anti-inflammatory agents, there is no laboratory method for the approximate determination of their metabolities in urine. Such an examination may be of value since it has been suggested (1) that the metabolism of salicylates can vary in disease. Urinary results would, however, be affected by differential treatment of the various metabolites by the kidney (2).

It is now generally agreed that following the oral administration of salicylates the chief metabolites found in the urine are free salicylic and gentistic acids and conjugates such as salicyluric acid.

The following technic permits an approximate evaluation of the metabolites of salicylic acid after large oral doses, and can be carried out in the clinical laboratory. Single dimensional paper chromatography is employed and only about 5 ml. of urine are required. The benzene-acetic acid-water system of Bray, Thorpe, and White (3) and the Kawerau Unit (4) are used. The latter, because of its small size, permits ready temperature control. The most sensitive reaction for the determination of phenolic compounds such as salicylic acid and gentistic acid is the phenol reagent of Folin and Ciocalteu (5). This reagent, however, is nonspecific, the reaction being given by many nonphenolic substances normally present in urine. When examined by the chromatographic system of Bray *et al.* (3), it has been found that these nonspecific substances remain stationary, whereas gentistic and salicylic acids are separated. Salicylic acid has a high R_f value, whereas gentistic and salicyluric acids have low R_f values (Fig. 1*A*).

The complete evaluation is made from two runs. In the first, 100

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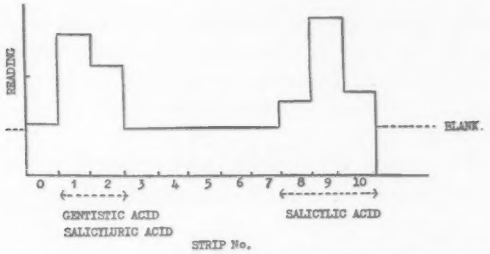

Fig. 1*A*. R_f values of gentistic, salicyluric, and salicylic acids.

μl. of urine are applied to the paper. In the second run, 100 μl. of urine are applied after acid hydrolysis. For this purpose, 0.4 ml. of concentrated hydrochloric acid is added to 5 ml. of urine in a stoppered tube and placed in a boiling water bath for 30 minutes. After cooling, the contents are made up to volume with water. In the present study, a number of assumptions have been made:

1. *Unhydrolysed urine.* High-R_f phenolic material represents free salicylic acid. This is not strictly true since other free phenols would be included, but they are usually negligible.
2. *Hydrolysed urine.* The increase in high-R_f phenolic material represents conjugated salicylic acid. Other phenol conjugates will also be included but these usually only amount to about 50 mg. (as phenol) per day.
3. *Hydrolysed urine.* Low-R_f phenolic material material represents gentistic acid, free and conjugated.
4. There is no destruction as the result of acid hydrolysis.

The quantitative determination of salicylic and gentistic acids from the chromatograms necessitates reference to standards since each has a different sensitivity toward the phenol reagent. Experiments have been

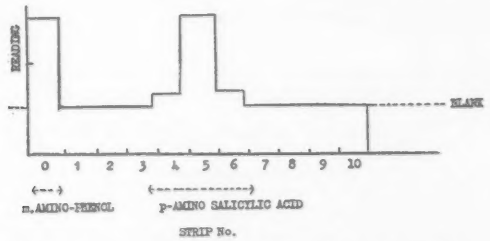

Fig. 1*B*. R_f values of *p*-amino salicylic acid and *m*-amino phenol.

carried out with regard to possible interference from *p*-amino salicylic acid. Reference to the Fig. 1B shows that *p*-amino salicylic acid has an R_f intermediate between gentistic and salicylic acids and *m*-amino phenol; the product produced by the action of hot dilute mineral acids does not move in the system.

Quantities of gentistic and salicylic acid (10–50 μg.) added to paper and subjected to the technic could be recovered with an accuracy of 78–108 per cent.

METHOD

Reagents

1. Folin-Ciocalteu phenol reagent (5).
2. Sodium carbonate, 5N solution.

Apparatus

1. Whatman No. 1, filter paper strips 20 mm. wide.
2. Kawerau unit.

Two parts of benzene, 2 parts of glacial acetic acid, and 1 part of water are shaken in a separating funnel and the phases allowed to separate. The phases are separated and placed in each of the two flasks.

Procedure

On a strip of Whatman No. 1 filter paper 20 mm. wide, an area 25 mm. long is marked with pencil lines. Urine (untreated or hydrolysed) is applied within this area and allowed to dry until a total volume of 100 μl. have been added. This area is designated as 0. The paper is arranged within the Kawerau unit so that one end dips into the benzene phase and the other is free. Area 0 is located just above the benzene phase. The solvent is allowed to run up the paper until it has advanced about 250 mm. beyond Area 0 (6–8 hours). The paper is removed, the position of the solvent front marked and the paper allowed to dry at room temperature. The end containing and including Area 0 is cut off and discarded. The

Table 1. Urinary Excretion of Metabolites of Salicylic Acid after Oral Administration in Rheumatic Fever

Case No.	*Free salicylic acid* (mg./100 ml.)	*Salicylic acid conjugates* (mg./100 ml.)	*Gentistic acid* (free and conjugated) (mg./100 ml.)
1	114 (47.9%)[a]	40 (16.8%)	84 (35.3%)
2	194 (68.4%)	45 (15.8%)	46 (15.8%)
3	230 (72.7%)	nil (0%)	86 (27.3%)
4	92 (43.8%)	52 (25.2%)	66 (31.0%)

[a] The figures in parentheses refer to the percentage of the total metabolites excreted.

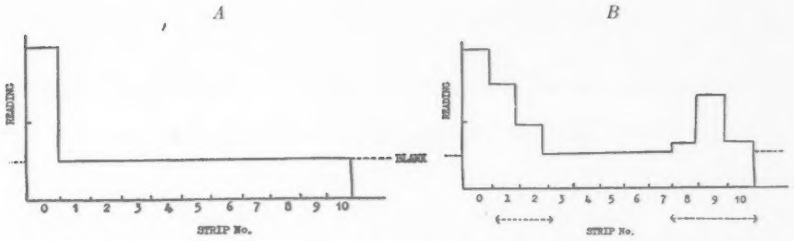

Fig. 2*A* and *B*. R_f values of normal urine compared to urine after salicylate administration.

remainder of the strip up to the advance of the solvent front is divided into 10 equal parts. These are numbered from 1 to 10 and correspond approximately to R_f values of 0–0.1, 0.1–0.2, and so on to 0.9–1.0 (Fig. 2*A*).

These pieces of paper are each placed in 5 ml. of water. An 0.25-ml. amount of Folin-Ciocalteu reagent is added, followed by 1 ml. of 5N sodium carbonate solution. The tubes are allowed to stand at room temperature for 30 seconds and then placed in a boiling water bath for 1 minute. After cooling the colors are read against water in a Unicam spectrophotometer S.P. 350 at 600 mμ.

The results may then be plotted in the form of a graph (Fig. 2*B*).

Calculation

The average reading obtained for Strips 4, 5, and 6 is taken to represent the blank, which is then subtracted from each of the other strip readings. The readings will be as follows:

1. *Unhydrolysed urine.* The corrected readings for Strips 8, 9, and 10 represent free salicylic acid.
2. *Hydrolysed urine.* (a) The corrected readings for Strips 8, 9, and 10 represent total salicylic acid (free and conjugated); (b) the corrected readings for Strips 1, 2, and 3 represent total gentistic acid (free and conjugated).

Examination of Urine after Oral Administration of Salicylates

Results are shown in the Table 1 and in Fig. 2*B*.

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Absorption and Excretion of 17, 21-Dihydroxy-20-Ketosteroids in Dogs

Robert H. Silber and Evan R. Morgan

STEROID ANALYSES performed after intravenous administration of solutions of hydrocortisone and cortisone to man have established the plasma half-life of hydrocortisone to be 2 hours and that of cortisone 1 hour (1, 2). From isotopic determinations in urine, the half-life of hydrocortisone in the body has been found to be 6 hours and that of cortisone 3.6 hours (3, 4). Thus, cortisone is not only cleared from the plasma more rapidly than hydrocortisone but it is also metabolized and excreted at a faster rate. In rats (5), hydrocortisone acetate has been shown to be very slowly absorbed from injection sites, whereas hydrocortisone is more rapidly absorbed than cortisone, cortisone acetate, or hydrocortisone acetate. In these studies it was shown that biologic activity was dependent upon the rate of absorption of the steroids from the injection sites.

It therefore seemed desirable to compare the absorption, excretion, and half-life in plasma of several steroids including the newer forms, 9α-fluorohydrocortisone, prednisone, and prednisolone. Information regarding the mechanism responsible for the greater biologic activity of these steroids might be obtained in such studies.

EXPERIMENTAL

Adult fasted beagle dogs weighing 12–14 Kg. were used. The analytical procedure was that of Silber and Porter (6) except for the use of a higher concentration of β-glucuronidase as noted elsewhere (7).

For intramuscular administration the steroids were suspended in saline (50 mg./ml.) and for oral administration, they were dissolved in 10–20 ml. of ethanol, diluted with 50 ml. of water, and the stomach tube

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was washed with additional water. For intravenous administration, the steroids were dissolved in alcohol (25 mg./5 ml.) and diluted to 125 ml. with saline. The infusion of 25 mg. of steroid was completed in a 15-minute period.

RESULTS

Plasma Concentrations

Intramuscular Injection

After intramuscular administration of 100 mg., plasma samples taken after 1, 3 and 5 hours revealed increases in steroid concentration of 8–14 μg./100 ml. after administration of hydrocortisone, cortisone, cortisone acetate, prednisone, and prednisolone without a peak in the 5-hour period. Hydrocortisone acetate and 9α-fluorohydrocortisone injection failed to cause a measurable increase in the plasma steroid concentration.

Oral Administration

After oral administration of 100 mg. striking differences in the plasma concentrations were found (Fig. 1). Prednisolone concentrations were far greater than those observed with the other 6 steroids and the plasma concentrations of the two Δ^1 steroids were more prolonged. 9α-Fluorohy-

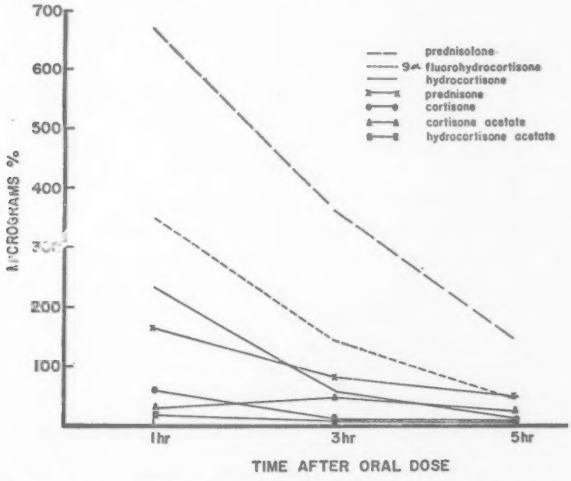

Fig. 1. Plasma concentrations after 100 mg. orally (increase in μg. per cent; averages of 2 dogs).

drocortisone showed the next highest peak value. Concentrations found after administration of cortisone, cortisone acetate, and hydrocortisone acetate were lowest.

Intravenous Injection

After intravenous administration of 25 mg., peak concentrations determined 5 minutes after the infusions ranged from 135–235 μg. per cent. The curves and the half-life times are shown in Fig. 2. 9α-Fluorohydrocortisone and prednisolone had slightly longer half-life times than hydrocortisone, and prednisone had a longer half-life than cortisone, which was the most rapidly cleared of the steroids studied.

Urinary Excretion

The urinary excretion data are summarized in Table 1. The steroids are listed in decreasing order, based upon their total excretion after oral administration, with hydrocortisone at the top and 9α-fluorohydrocortisone at the bottom. After intramuscular injection, relatively little steroid was found, with almost negligible amounts excreted after giving all except hydrocortisone and prednisolone. After intravenous infusion, 14 per cent of the dose of hydrocortisone was found, and 10.6 per cent after giving its Δ^1 derivative. From 5 to 7.5 per cent of each of the other three steroids was detected in the urine.

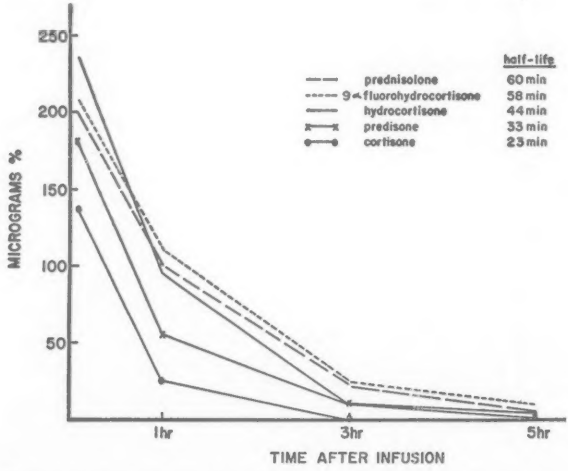

Fig. 2. Plasma concentrations after 25 mg. intravenously (increase in μg. per cent; averages of 2 dogs).

Table 1. URINARY EXCRETION AFTER ORAL, INTRAMUSCULAR, AND INTRAVENOUS ADMINISTRATION

	Oral, 100 mg.		*I. M., 100 mg.*		*I. V., 25 mg.*	
	Free	*Conjugated*	*Free*	*Conjugated*	*Free*	*Conjugated*
Hydrocortisone	2.5[a]	22.0	1.05	4.0	0.7	2.8
Prednisolone	6.2	1.9	0.9	0.4	2.2	0.45
Hydrocortisone acetate	1.9	5.2	0.17	0.11	..	..
Cortisone	1.65	5.25	0.75	0.65	0.4	0.9
Cortisone acetate	2.1	3.1	0.22	0.19	..	..
Prednisone	3.5	1.0	0.8	0	1.2	0.3
9α-Fluorohydrocortisone	2.7	1.3	0.33	0.24	0.9	1.0

NOTE: It has been found that tetrahydrohydrocortisone is essentially the sole glucuronide form that is excreted after giving hydrocortisone to dogs, so the values listed in the "conjugated" column have been corrected for the incomplete recovery of this steroid in the procedure used.

[a] All results are the averages found with 2 dogs, expressed in mg./24 hr.

The total excretion of 17,21-dihydroxy-20-ketosteroids after oral administration of 100 mg. hydrocortisone to 2 dogs (average 24.5 mg.) was not significantly different from the excretion observed after intravenous administration of the same amount (average 25.2 mg.).

Normal dogs excrete very small amounts of 17,21-dihydroxy-20-ketosteroids, about 100–300 μg. in 24 hours, so corrections for the basal excretion have not been made in this paper.

DISCUSSION

The excretion of conjugated forms of prednisone, prednisolone, and 9α-fluorohydrocortisone after oral administration was obviously depressed as a result of the introduction of the Δ^1 double bond or the fluorine into the molecule. This indicates that reduction of these forms in the 3 position is inhibited, thereby depressing the rate of conjugation. Since reduction of the A ring is a major inactivation process in the body, it seems likely that retardation of this process is at least partially responsible for the greater biologic activity of these three steroids. Lower excretion of the glucuronide forms of the three steroids was also evident after intravenous or intramuscular administration.

Since it has been found (7) that dogs, like man, excrete a constant fraction of a given oral dose of hydrocortisone, the excretion data in Table 1 should make it possible to estimate the fraction of an intramuscular or oral dose that has been absorbed. Thus, since about 25 per cent of the oral hydrocortisone dose was excreted in the urine as 17,21-dihydroxy-20-ketosteroids, excretion of 5 mg. after intramuscular in-

jection of 100 mg. indicates that 20 mg., or 20 per cent, were absorbed in the 24-hour period. Similar calculations reveal that 16–20 per cent of the intramuscular doses of cortisone, prednisolone, and prednisone were absorbed from the injection sites in the same time. Furthermore, if one makes a correction for the basal excretion of 0.2 mg. per dog per day, such calculations indicate that about 1 per cent of the hydrocortisone acetate, 4 per cent of the cortisone acetate, and 10 per cent of the 9α-fluorohydrocortisone were absorbed in the 24 hours after injection.

The plasma half-life of the steroids with increased biologic activity was measurably but not strikingly prolonged after intravenous administration. After oral administration, plasma concentrations were not only higher but also more prolonged than after administration of hydrocortisone or cortisone. These results support the excretion data, which indicated that the metabolic inactivation of prednisone, prednisolone, and 9α-fluorohydrocortisone was depressed.

The plasma half-life times were calculated after plotting the logarithm of the increase in plasma concentration against time. As noted in man after intravenous infusion (1, 2), such a plot yields a straight line. It is of interest that a straight line is also obtained when the values obtained after oral administration are plotted in the same way.

SUMMARY

The plasma concentrations of free 17,21-dihydroxy-20-ketosteroids and urinary excretion of both free and glucuronide forms have been determined after oral, intramuscular, and intravenous administration of cortisone, hydrocortisone, their Δ^1 forms, and 9α-fluorohydrocortisone to dogs.

Excretion of the glucuronide forms of those steroids with a double bond at the 1–2 position or with a fluorine at position 9 was depressed, and the plasma half-life of each of these steroids was longer than that of the parent compound.

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Serum Iron Determination

George R. Kingsley and Gloria Getchell

AN IRON REAGENT, 4,7-diphenyl-1,10-phenanthroline, which has a more desirable sensitivity than other known iron reagents, has been introduced by Case (1), has been used by Smith *et al.* (2) in the estimation of iron in water, and in serum by Peterson (3) who reported the molar absorbency index of the iron complex of this new reagent as almost three times that of α-α'-dipyridyl or *o*-phenanthroline. This reagent also has an added advantage in that its iron complex can be separated with isoamyl alcohol from aqueous solutions which contain interfering ions.

We have made a comparative study of several serum iron methods, and have obtained lower iron values with the method of Peterson (3) than that of Kitzes *et al.* (4) or Ramsey (5). Since the methods of Kitzes and Ramsey require larger serum specimens and are time consuming, and the method of Peterson gave lower serum iron values, an investigation was undertaken to develop an improved serum iron method using the more sensitive 4,7-diphenyl-1,10-phenanthroline reagent.

METHOD

Reagents

All reagents must be iron free.

1N Hydrochloric acid.

Hydrazine sulfate: A saturated solution of reagent-grade hydrazine sulfate in water. (This reagent is stable in an amber Pyrex bottle.)

Sodium acetate (reagent grade): Prepare a saturated solution in iron-free distilled water. If significant iron contamination is present in the

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sodium acetate it may be purified by adding a few drops of hydrazine sulfate and shaking with phenanthroline-isoamyl alcohol reagent.

Isoamyl alcohol (reagent grade).

Absolute ethyl alcohol (reagent grade).

Iron-free distilled water: Redistilled from all-glass still.

0.0025M Phenanthroline-Isoamyl alcohol reagent: Dissolve 70 mg. of 4,7-diphenyl-1,10-phenanthroline ($[C_6H_5]_2C_{12}H_6N_2$) (G. Frederick Smith Chemical Co.) in isoamyl alcohol and dilute to 500 ml. with isoamyl alcohol.

Stock Iron Standard

Thoroughly clean a small piece of iron wire of known iron content (usually 99.8 per cent). Weigh an amount of wire that contains approximately 100 mg. of iron. Place the iron in a 250-ml. Florence flask and add 10 ml. of concentrated iron free HNO_3 and about 40 ml. iron-free distilled water. Heat to boiling and boil until completely dissolved. Continue boiling 1 minute longer. Cool and transfer solution quantitatively to a 100-ml. volumetric flask. Dilute to volume with iron-free distilled water and mix; 1 ml. = 1 mg. Fe. This solution is stable indefinitely if kept free of contamination.

Dilute Iron Standard

Dilute 1 ml. stock iron standard to 1000 ml. with iron-free distilled water; 1 ml. = 1 μg. Fe.

Standardization

Accurately measure 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0 ml. of dilute iron standard into glass-stoppered test tubes (16 x 150 mm.). Dilute to 4 ml. with iron-free distilled water and continue as directed in PROCEDURE.

Calculation

Prepare a graph on semi-log paper by plotting per cent transmission (ordinate) against μg. concentration of iron (abscissa) per 100 ml. Concentration may also be calculated from the formula: μg. Fe per 100 ml. of serum = K[1] $(2 - \log \%T)$.

Collection of Serum

All serum specimens used in this investigation were collected under neutral, light, mineral oil in an acid-washed test tube (25% HCl). Slight hemolysis will increase serum iron values. Hemolysis is avoided

[1] K = 1400 with Coleman spectrophotometer model 6, with No. 6-304 B cuvets.

by using an airtight needle and a syringe oiled with light neutral mineral oil. After removing the needle, add blood directly from the syringe to a test tube, allowing blood to run gently down the side of the tube containing about 0.5–1.0 ml. of the mineral oil. Centrifuge the blood after it has been allowed to clot for 30–45 minutes. Separate the serum from the clot immediately after centrifugation.

Procedure

Pipet 1 ml. of fresh unhemolyzed serum into a glass-stoppered test tube (16 x 150 mm.). Add 3 ml. of distilled water and 2 ml. of 1N HCl. Prepare a reagent blank in the same manner, substituting 1 ml. of distilled water for 1 ml. of serum. Heat tubes in boiling water bath for 30 minutes. Cool, add 2 ml. of saturated sodium acetate, 6 ml. of phenanthroline-isoamyl alcohol reagent, and 0.5 ml. of saturated hydrazine sulfate. Stopper the test tubes and shake them in a Kahn shaker for 15 minutes. Remove stoppers and centrifuge. Pipet 5 ml. of the isoamyl alcohol layer into photometer cuvets. Add 0.5 ml. of absolute alcohol to clear the isoamyl alcohol extract, and mix. Read at 530 mμ against the reagent blank set at 100% T.

EXPERIMENTAL

Reproducibility and Normal Range of Method

Maximum absorbence of the ferrous complex of 4,7-diphenyl-1,10-phenanthroline occurs at 530 mμ (Fig. 1). The effect of reducing agents

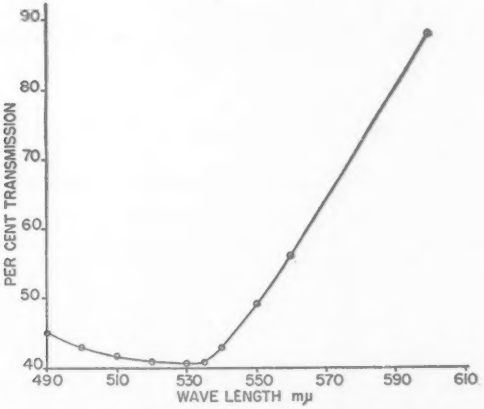

Fig. 1. Absorption spectra of the ferrous complex of 4,7-diphenyl-1,10-phenanthroline as obtained with the Coleman Junior Spectrophotometer, No. 6, using 304 B cuvets.

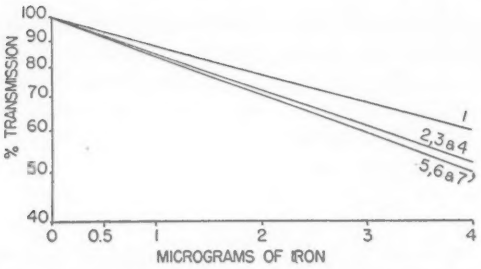

Fig. 2. Effect of different reducing agents on the standardization curve of the ferrous complex of 4,7-diphenyl-1,10-phenanthroline as obtained with the Coleman Junior Spectrophotometer No. 6, using 304 B cuvets. *Curve 1*, sodium sulfite. *Curves 2, 3, and 4*, hydroquinone, hydrogen, and thioglycolic acid. *Curves 5, 6, and 7*, phenylhydrazine, hydrazine sulfate, and ascorbic acid.

on the standardization curve of the ferrous complex is shown in Fig. 2. 0.5 ml. of aqueous solutions of 21.0% Na_2SO_3, 5.0 per cent hydroquinone in 1N HCl, 5.0 per cent ascorbic acid in 1N acetic acid, saturated hydrazine sulfate, 5.0 per cent phenylhydrazine, and 2 drops of Eastman thioglycolic acid were added with the phenanthroline-isoamyl alcohol-iron reagent. Hydrogen from a zinc-HCl generator was bubbled into the iron standards (after HCl digestion) through a fritted gas-dispersion cylinder (12 mm. coarse, No. 39533 tube, borosilicate glass, Corning). Phenylhydrazine, hydrazine sulfate, and ascorbic acid apparently have about the same activity in the reduction of iron in this procedure. Heilmeyer (6) has obtained 30 per cent more serum iron with hydrogen

Table 1. Total Serum Iron Recoveries with the 4,7-Diphenyl-1,10-Phenanthroline Method

Serum iron (μg. %)	*Iron added* (μg. %)	*Iron recovered* (μg. %)	*% Recovery*
113	50	163	100
172	50	215	97
160	100	250	96
150	100	240	96
195	100	295	100
82	150	216	93
150	200	344	97
122	200	322	100
166	200	350	96
122	200	350	108
59	250	300	98
173	250	437	103
88	300	380	98

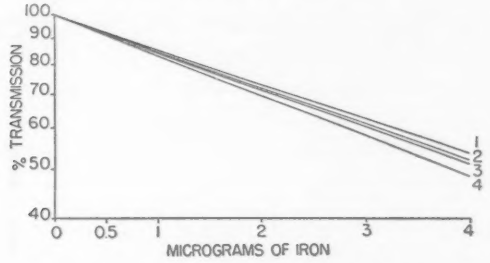

Fig. 3. Effect of different reducing agents in the presence of serum on the standardization curve of the ferrous complex of 4,7-diphenyl-1,10-phenanthroline as obtained with the Coleman Junior Spectrophotometer No. 6, using 304 B cuvets. *Curve 1*, phenylhydrazine. *Curve 2*, ascorbic acid, hydroquinone, and thioglycolic acid. *Curve 3*, hydrogen, and sodium sulfite. *Curve 4*, hydrazine sulfate.

reduction than with hydroquinone when phenanthroline was used as the iron reagent.

Good recoveries of total serum iron were obtained with the 4,7-diphenyl-1,10-phenanthroline reagent (Table 1). The different reducing agents produced different shades of color in the reduction of iron from the ferrous complex of 4,7-diphenyl-1,10-phenanthroline. A pink-red color was produced with sodium sulfite, hydrogen, and thioglycolic acid; orange-pink color with hydrazine sulfate, ascorbic acid, and hydroquinone; and yellow-orange color with phenylhydrazine. In spite of the different shades of color, maximum absorbence with all the reducing agents was obtained at 530 mμ. In the presence of serum, hydrazine sulfate gave a color with the greatest light absorbence (Fig. 3). This reagent is the most stable and convenient to prepare of the reducing agents used.

Comparison with α-α'-Dipyridyl Method

The 4,7-diphenyl-1,10-phenanthroline serum iron method was applied directly to normal serum as described in PROCEDURE and also to dry-ash residues of aliquots of these serums and compared to the α-α'-dipyridyl method of Kitzes *et al.* (4) (Table 2). The method of Kitzes was slightly modified by using Na_2SO_3 as described by Delory (7) instead of thioglycolic acid. The method of Peterson (3) gave values somewhat lower than those obtained by the method of Kitzes, which might be expected, as acid hydrolysis of serum is not specified in the method of Peterson.

Table 2. Comparison of 4,7-Diphenyl-1,10-Phenanthroline and α-α′-Dipyridyl Methods in the Determination of Serum Iron
Normal Individuals 20–60 Years Old

Case no.	4,7-Diphenyl-1,10-phenanthroline		α-α′-Dipyridyl (acid digestion) (μg. %)
	Acid digestion (μg. %)	Dry ash (μg. %)	
Females			
1	185	210	125
2	185	205	..
3	160	160	..
4	200	200	110
5	160	195	100
6	120	130	95
7	190	195	170
8	125	140	110
9	200	220	170
10	135	..	105
11	138	..	105
12	125	..	105
Average	160	..	..
Males			
1	210	220	..
2	238	240	115
3	170	170	160
4	230	220	200
5	200	200	180
6	175	190	150
7	158	..	145
8	125	..	82
9	125	..	82
10	216	..	180
11	215	..	180
12	169	..	115
13	134	..	110
Average	182		142

Dry Ashing

The dry ashing of the serum was carried out as follows:

1 ml. of serum in a 20-ml. beaker is dried in a forced-draft oven at 100° for 4 hours or until free of moisture. The specimen is ashed in a muffle furnace for 2 hours at 200°. The temperature is then raised to 500° and maintained at this temperature for about 12 hours or until free of carbon. The ashed residue is dissolved in 2 ml. of distilled water, 1 ml. of HCl, and 1 drop of concentrated HNO_3 and evaporated to dryness on an electric hot plate. The ash is dissolved in 2 ml. of distilled

water and evaporated to dryness. The ash is again dissolved in 2 ml. of distilled water and evaporated to dryness, and the residue transferred to a glass-stoppered test tube with 3 ml. of distilled water and 2 ml. of 1N HCl. The tube is placed in a boiling water bath for 30 minutes and the determination continued as described in PROCEDURE.

Acid Digestion

Good agreement between the acid-digestion and dry-ashing procedures was obtained with the diphenyl-phenanthroline reagent (see Table 2). The dry-ashing procedure averaged 4.6 per cent higher than acid digestion. It has been reported (8) that iron fraction D completely resists extraction by HCl and can only be obtained by total incineration of the serum. This fraction was found by these investigators (8) to average 16 to 17 per cent of the total iron. However, the incineration values (296 μg./100 ml. [male] and 242 μg./100 ml. [female]) which these investigators obtained were considerably higher than our incineration values.

α-α′-Dipyridyl

Considerably lower serum iron levels were obtained with the α-α′-dipyridyl method (see Table 2), in spite of fact that acid hydrolysis was carried out in the same manner in both procedures. The normal range of serum iron as determined by the 4,7-diphenyl-1,10-phenanthroline method ranged in the female from 120 to 200 μg./100 ml., averaging 160 μg./100 ml., and in the male from 125 to 238, averaging

Table 3. COMPARISON OF THE 4,7-DIPHENYL-1,10-PHENANTHROLINE AND α-α′-DIPYRIDYL SERUM IRON METHODS
Patients 20–70 Years Old with Various Clinical Disorders

Clinical disorder	*No. cases*	*4,7-diphenyl-1,10 phenanthroline* (μg. %)	*Dipyridyl* (μg. %)
Anemia			
Nutritional macrocytic	4	138–232	125–200
Chronic hemolytic	2	380–400	312
Aplastic	1	313	270
Myelofibrosis	1	198	160
Undetermined etiology	5	60–120	37–82
Hemochromatosis	5	140–263	120–238
Cirrhosis	1	377	313
Hepatitis	1	300	200
Miscellaneous (Atherosclerosis, hypertension, subacute bacterial endocarditis, reticulum-cell sarcoma, chronic pancreatitis with diabetes)	5	63–215	40–187

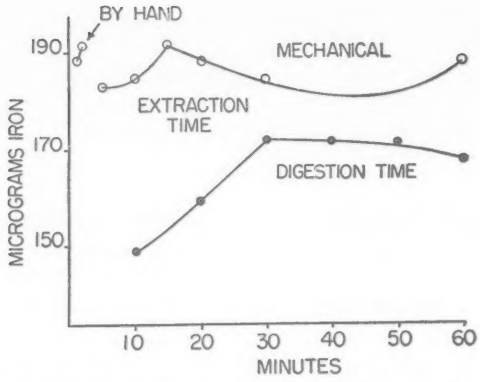

Fig. 4. Determination of the optimum digestion and extraction time required for the release of iron from pooled sera when hydrazine sulfate is used as a reducing agent.

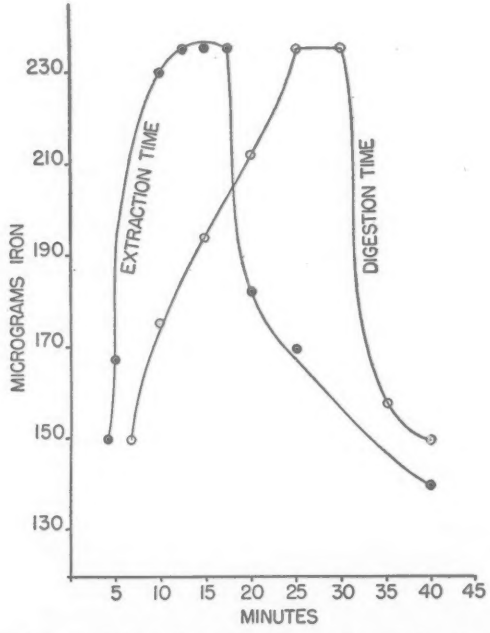

Fig. 5. Determination of the optimum digestion and extraction time required for the release of iron from pooled sera when thioglycolic acid is used as a reducing agent.

182 μg./100 ml. The α-α′-dipyridyl method averaged 40 μg./100 ml. lower in both sexes than the 4,7-diphenyl-1,10-phenanthroline method. Similar differences in serum iron concentrations between the two methods were also obtained in patients (Table 3).

Determination of Optimum Digestion and Extraction Time

Data from several single and pooled serums indicated that the optimum digestion time of serum was found to range between 30 and 35 minutes (Fig. 4). We have found that occasionally some sera do require only 15 to 25 minutes digestion but no loss of iron occurs if digestion is continued up to 40–60 minutes in these sera. We were not able to obtain optimum recovery of serum iron by digesting 10 to 15 minutes at 90–95° with 20% trichloracetic-thioglycolic acid reagent as recommended by Peterson (3). Complete extraction of iron from serum can be obtained by a vigorous 2-minute hand shaking or in approximately fifteen minutes with a mechanical shaker. Both extraction and digestion times were quite critical if thioglycolic acid reagent (3) was used instead of hydrazine sulfate (Fig. 5).

SUMMARY

1. A sensitive, reproducible method for serum iron determination has been developed employing extraction of serum acid hydrolysate with isoamyl alcohol and development of a highly colored ferrous complex of 4,7-diphenyl-1,10-phenanthroline for quantitative photometric measurement of iron.

2. The method gives good recoveries and also good agreement with the dry-ashing procedure.

3. A normal range of 120–200 μg./100 ml. (average 160) for the female and 125–238 μg./100 ml. (average 182) for the male for serum iron was obtained by the method.

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Urinary Estrogens in Pregnancy

Improved Method for Their Determination in Humans

Rudolph M. Anker

ONE OF THE DIFFICULTIES encountered in the determination of urinary estrogens arises from the fact that no procedure so far developed is capable of cleaving the estrogen conjugates quantitatively without destroying any of the free steroids (1). Reporting on a thorough investigation of this subject Van Bruggen (2) stated: "The use of butanol as the solvent and hydrolyzing medium . . . appears to be of definite promise." Following this suggestion, an improved method has been developed for the determination of estrogens in the urine of pregnant women. This method yields estrogen values 10–30 per cent higher than those obtained by conventional methods.

REAGENTS

The *n*-butanol to be used must be tested for the presence of impurities which destroy the estrogens during hydrolysis. Reagent-grade butanol cannot be assumed to be free of such impurities. Butanol of satisfactory quality was prepared by refluxing 1 L. of butanol with 1 Gm. of *o*-phenylene diamine and 50 ml. of concentrated HCl for 24 hours. The butanol was then distilled off in a current of steam.

METHODS

Extraction

An aliquot of the urine specimen, about 500 ml., is acidified to approximately *p*H 2 with phosphoric acid, saturated with butanol, and placed

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in tube *A* of the tube-air lift extractor shown in Fig. 1. The butanol (50 ml.) is added at *B*, the stopper (*C*) is placed in position, the tube (*D*) being adjusted to just touch the upper surface of the butanol in *A*. A slow current of air is introduced through the right-angle tube (*E*), which escapes via *D*, carrying with it drops of butanol. The rate of flow of the butanol is regulated by the screw clamp (*F*) to the maximum attainable without causing an emulsion to form in the upper part of column *A*. Approximately twenty minutes are required to attain 99 per cent of the equilibrium concentration of methyl red in the butanol phase. In the case of the estrogen conjugates 45 minutes are allowed for equilibration. Finally all butanol is collected in *A* by removing *C*, *D*, and *E* and applying air pressure at *B*. The butanol is drawn off as completely as possible by pipet.

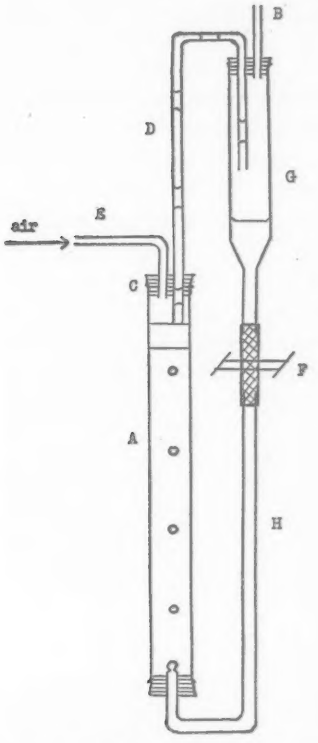

Fig. 1. Tube-air lift extractor. Tube *A* should have a diameter of approximately 30 mm. Tube *D* should have an inside diameter of no more than 3 mm. and may be constructed of capillary tubing for extra sturdiness. The section *G* should be at least 18 mm. wide, with section *H* having an inside diameter of approximately 5 mm.

The urine is extracted five times, using 50 ml. of *n*-butanol saturated with water for each extraction. The extracted urine is discarded. The acidity of the aqueous layer is checked after each extraction and adjusted to approximately *p*H 2 by adding further quantities of phosphoric acid. Although occasional urine specimens form emulsions involving part or all of the butanol layer unless the extractor is run extremely slowly, emulsion formation can be disregarded in adjusting the flow rate, if the emulsion is removed and filtered at the end of the first extraction. The aqueous layer of this filtrate is returned to the extractor, and the butanol layer is combined with the unemulsified butanol, if any. Emulsions

Table 1. Comparison of Results of Aqueous- and Butanolic-Hydrochloric Acid Methods of Hydrolysis in Urinary-Estrogen Determination

	Urine sample									
	A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*K*
Aqueous hydrochloric acid	6.65[a]	21.4	9.7	8.6	9.4	1.05	9.1	7.6	14.0	6.6
Butanolic hydrochloric acid	7.55	27.9	11.3	10.5	10.4	0.91	9.9	11.4	28.0	12.3

[a] All values are in milligrams per liter.

will not be formed in subsequent extractions, because this one filtration removes the emulsion-stabilizing solids from the urine.

Separation and Assay

The combined butanol extracts are heated to boiling under reflux. Concentrated HCl is added through the condenser, in an amount to equal 15 per cent of the combined solvent extract, and the refluxing is continued for 1 hour. After cooling, the acidified butanol is brought to approximately *p*H 7 by the slow and careful addition of concentrated NH_4OH. The butanol is distilled off in a vigorous current of steam, and by supplying additional heat from a burner. The volume of the resulting aqueous solution is arranged to be 50 to 100 per cent of that of the butanol originally present. To the warm aqueous solution, toluene, in amounts equal to one tenth the volume of the aqueous solution, is added, and the contents of the distilling flask are transferred quantitatively to a separatory funnel. Small particles of tarry matter are dissolved in a few milliliters of ethanol. The estrogens are then concentrated and assayed as described previously (3).

RESULTS

The estrogen values obtained were higher than the values obtained by the previous methods (3), with the exception of Sample F, which was from a patient with severe preeclampsia and very low estrogen output (Table 1).

DISCUSSION AND CONCLUSIONS

Venning (4) and Stimmel (5) have shown that the conjugated estrogens in urines of pregnant women can be extracted quantitatively with four portions of one-tenth volume of butanol from urines acidified to Congo red. This corresponds generally with our experience; but a qualification

needs to be made, which applies to all deductions based on experiments with urinary estrogens—as the properties of the conjugated estrogens vary considerably from one urine sample to another, something like a dozen urine specimens from different individuals should be tested, preferably including cases with abnormalities such as toxemia of pregnancy, before a given method can be accepted as consistently reliable. It needs to be specified further that the urines extracted with butanol as described above did not contain any estrogens as determined by the Allen-Kober test (6). We have encountered one urine specimen that retained 4 per cent of its estrogens after four extractions with one-tenth volume of butanol, and we now use five extractions routinely.

Van Bruggen (2) first called attention to the possible advantage of hydrolyzing the conjugated estrogens in a medium of butanol. This has been confirmed. The cleavage in butanol has been found to be complete after 60 minutes' boiling with acid in all urines tested so far, while complete cleavage of the conjugates in the same urines, in an aqueous medium, required boiling for 90 minutes or more. Also, the free steroids are more stable in hot butanolic acid than in aqueous acid, the rate of destruction of estrogens in butanol being about 1 per cent per hour versus 2–6 per cent in aqueous acid.

Extraction with butanol and hydrolysis in this solvent, therefore, seem to be quite efficient operations. The reason that they have not been more widely adopted seems to be the frequent occurrence of very stable emulsions of butanol and urine at *p*H 2, which have to be broken by centrifugation, a time-consuming step. The need for this has now been eliminated by the use of the tube-air lift extractor.

SUMMARY

A new method has been described for the cleavage of the estrogenic steroid conjugates occurring in the urine of pregnant women. It yields higher estrogen values than the methods which have been employed in the past.

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Vanadium Concentration of Urine

Rapid Colorimetric Method for Its Estimation

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VANADIUM COMPOUNDS are used in dye, ink, and glass manufacture, as an alloy component in the steel industry, and as a catalyst in the production of sulfuric acid. Toxic exposures have been reported in the extraction of vanadium from ores and residues (1–5) and in the cleaning of burners fired with vanadium-bearing oil (6–8). The outstanding symptom associated with excessive inhalation of vanadium compounds, particularly the pentavalent forms, is acute irritation of the respiratory tract, sometimes attended by chemical pneumonitis (5). Irritation of the conjunctivae and eczematous lesions of the skin may also occur. Exposure to low levels of vanadium may cause metabolic disturbances as evidenced by a reduction in the fingernail cystine content (9).

Talvitie and Wagner (10) have shown that urinary vanadium concentrations constitute a reliable index to the degree of absorption of vanadium. Although vanadium in urine may be determined accurately with the method described by Talvitie (11), the need arose in toxicologic investigations for a faster screening test to provide pilot information on the absorption of vanadium. For this purpose a simple, colorimetric test for vanadium in urine was devised.

Necessary requirements for the test were (a) that only a small amount of urine be consumed in order to conserve the bulk of the sample for more accurate analysis, and (b) that the sensitivity be sufficient to detect minimal concentrations of vanadium. These conditions were met by basing the test on the catalytic effect of vanadium on the oxidation of organic chromogens by potassium chlorate. Szebelledy and Ajtai (12)

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describe several of these catalytic reactions. Because its oxidized form has a deep, magenta color easily distinguished in the presence of urine, *N*,*N*-diethyl-*p*-phenylenediamine was selected as the chromogen.

REAGENTS

Potassium Chlorate: Saturated solution.

Color Reagent: 1% solution of *N*,*N*-diethyl-*p*-phenylenediamine (*p*-aminodiethylaniline) monohydrochloride (Eastman No. 1374) in glacial acetic acid. This reagent should be prepared fresh each day to avoid a high blank from air oxidation of the color agent.

Standard Vanadium Solution: One milliliter contains 100 μg. of vanadium. Dissolve 0.2296 Gm. of ammonium metavanadate in 25 ml. of 4N H_2SO_4 and dilute to 1 L. with distilled water. Make dilutions with distilled water each time as needed.

PROCEDURE

1. Transfer a 1-ml. portion of each of the urine samples to be tested into test tubes large enough to contain at least 15 ml.
2. Prepare a control by measuring 1 ml. of the urine of an individual having no exposure to vanadium into another test tube.
3. Dilute the urine samples and control to 11 ml. with distilled water.
4. Add 1 ml. of saturated potassium chlorate solution to each test tube.
5. Add 1 ml. of the color reagent to each test tube, mix, and place the tubes simultaneously into a boiling water bath.
6. The test tubes may be observed during heating for development of a red-to-magenta color. Because vanadium-free urine also develops a slight reddish coloration, the presence of vanadium is indicated by color in excess of that found in the control.

After heating for 15 minutes, cool the tubes quickly to room temperature in a cold water bath. Additional color develops only slowly at room temperature and the colors are sufficiently stable to allow grading of the intensities.

7. The results may be quantified by comparing the samples with a simultaneously developed series of standards prepared by adding increments of standard vanadium solution to 1-ml. portions of vanadium-free urine. With a 15-minute heating period, a suitable color gradation is obtained with standards containing 0.0, 0.1, 0.2, 0.4, and 1.0 μg. of vanadium. The lower limit of sensitivity is 0.01 μg. of vanadium, whereas the colors developed by vanadium in excess of 1 μg. per ml. of urine (1 ppm) are so intense that they cannot be differentiated.

8. Numerical values may be assigned by reading the colors in a photometer at a wavelength of 540 mμ. The urine color may be conveniently compensated by reading the unknowns against duplicate samples prepared in the same manner as the unknowns except for the substitution of 1 ml. of distilled water for the potassium chlorate solution. A lower blank can be obtained by developing the colors at room temperature and after 1 hour of reaction sufficient sensitivity is obtained to cover the range from 0 to 1 μg. of vanadium per ml. of urine.

EXPERIMENTAL DATA

The method depends upon the catalytic effect of vanadium on the oxidation of *N*,*N*-diethyl-*p*-phenylenediamine by potassium chlorate. The rate of color development is proportional to the concentration of vanadium, but other factors which affect reaction rates, such as temperature, *p*H, and reagent concentrations, must be closely controlled if other than qualitative results are desired.

Volume of Urine

The optimum volume of urine for sensitivity was found to be 1 ml. Smaller volumes of urine tended to give higher blank values, whereas larger volumes of urine tended not only to mask the color but also to inhibit development of the color. In a series of urine: distilled-water dilutions of 1.0:9.0, 2.5:7.5, 5.0:5.0, 7.5:2.5, and 10.0:0.0 each containing the same amount of vanadium (1 μg. per sample) the most intense color was obtained with the 1.0:9.0 dilution. Also, when the urine from animals exposed to vanadium was tested, 10 ml. of undiluted urine gave only an amber color, while 1 ml. diluted to 10 ml. gave an intense magenta color. For convenience in measuring, the dilution in the test was standardized at 1 ml. of urine to 10 ml. of water.

Hydrogen-ion Concentration

The effect of *p*H on the color development was determined with a series of urine samples containing vanadium in which only the *p*H was varied with acetic acid-sodium acetate buffers. The *p*H values were determined with a Beckman Model G glass electrode *p*H meter. Table 1 shows that stable colors increasing in intensity with decreasing *p*H develop in the *p*H range of 2.0–3.7. Because the lowest blank was obtained at a *p*H of 2.6, the acidity was adjusted to this value. It was found that this value is obtained with sufficient accuracy with the glacial acetic acid contained in the color reagent. Because an excess of the acid has little additional influence on the *p*H, urine specimens in which a sediment has

Table 1. Effect of *p*H on Color Development of Urine Samples

*p*H	*Photoelectric colorimeter readings*[a]	
	Blank	*Test sample (0.1 μg. V/ml.)*
6.9	Colors faded to grey	..
5.6	Colors faded to grey	..
4.7	Partial fading	..
3.7	67	135
3.4	58	175
2.6	50	200
2.3	65	293
2.2	97	445
2.0	146	580

[a] Klett-Summerson photometer, with green filter #54.

formed may be clarified before the test by acidifying slightly with acetic acid.

Temperature and Time

The rate at which the color develops increases with temperature. At room temperature a color gradation suitable for colorimetric comparisons is obtained with concentrations varying from 0 to 1 μg. of vanadium per ml. of urine when the color is allowed to develop for 60 minutes. A hundredfold increase in rate of color development and sensitivity is obtained at the boiling temperature. The color continues to intensify as the heating time is extended beyond the 15 minutes specified in the procedure but the sensitivity is not increased materially thereby, because of a comparable increase in color in the control sample.

The color developed during the heating of the samples tends to fade slightly on cooling to room temperature. The relative intensities remain unaffected, however, and color comparisons can still be made 48 hours after removal of the samples from the water bath. If photometric readings are made, the time must be carefully standardized. The variations in readings with time at room temperature obtained with a Klett-Summerson photoelectric colorimeter are illustrated in Fig. 1. Qualitative comparisons or comparisons with a series of standards may be made at any time during the color development.

Specificity

Several metal ions are known to have a catalytic action similar to that of vanadium. Because of the possibility that these ions might appear in

borne vanadium compounds by man, dogs repeatedly exposed daily for several months by inhalation to vanadium pentoxide dust at the suggested threshold limit value (14) of 0.5 mg. of vanadium per cubic meter of air were found to have a urinary excretion level of about 1 μg. of vanadium per ml. of urine. If a similar response obtains in man, this test would have sufficient sensitivity to detect exposures of individuals to air-borne vanadium at or above the suggested threshold limit value.

SUMMARY

A rapid, convenient test for the estimation of small amounts of vanadium in urine is described. The method is based on the catalytic effect of vanadium on the oxidation of *N*,*N*-diethyl-*p*-phenylenediamine by potassium chlorate. Only 1 ml. of urine is required and the test is sensitive to quantities of vanadium as low as 0.01 μg. per ml. of urine. Semiquantitative analyses can be made over a range of 0–1 μg. of vanadium per ml. of urine. The test is designed for application in rapid clinical analyses on small amounts of urine to determine possible vanadium absorption and is suggested for use as a screening test to determine on-the-job exposure of workers to vanadium compounds in excess of the suggested threshold limit value. Data showing the application of the test in several species are given.

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Quantitative Nephelometric Microdetermination of Alpha-Globulins in Serum and Cerebrospinal Fluid

Abraham Saifer and Michael C. Zymaris

REVIEW OF METHODS

METHODS FOR THE QUANTITATIVE DETERMINATION of alpha-globulins in either blood or cerebrospinal fluid fall into three general categories: (a) alcohol or salt-fractionation technics, (b) electrophoretic analyses and (c) precipitation reactions with cationic detergents at controlled *p*H. Salt-fractionation procedures for α-globulins using sodium sulfate precipitation at a fixed concentration are those of Wolfson and Cohn (1) and of Kibrick and Blonstein (2). These clinical methods are suitable only for the high-protein concentrations found in serum, and both attempt to relate the chemical values to those obtained electrophoretically. While consistent checks between the two procedures can be obtained with normal sera, the careful experimental studies of Svensson (3) and Majoor (4) have shown that protein fractions precipitated at a single salt concentration *do not correspond* to those determined electrophoretically, especially for pathologic sera (5). The low temperature–low ionic strength fractionation methods of Cohn, Edsall, and their associates (6) have been extended by Lever *et al.* (7) to the small-scale fractionation of 5-ml. plasma samples. While such procedures are not truly quantitative, in that only about 75 per cent of total plasma protein

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is accounted for, they do serve to separate α- and β-lipoprotein-containing plasma fractions which have clinical significance in the study of various diseases (8, 9). These methods are laborious and unsuited to routine clinical work as well as being inapplicable for the analysis of cerebrospinal fluid.

Both the Tiselius moving-boundary method (10, 11) and the more recent paper electrophoretic technic (12, 13) have been applied to the quantitative determination of protein fractions, including α-globulins, in serum (14, 15) and cerebrospinal fluid (16, 17). For the latter fluid, because of its low protein content, the use of the moving-boundary method requires the use of expensive microelectrophoretic equipment with increased measuring sensitivity based on interferometric methods (18). Both technics require the time-consuming step of concentrating large volumes (about 10 ml. of cerebrospinal fluid to about 0.1 ml.), prior to the analysis. Aside from the high cost or lack of availability of such equipment, neither technic lends itself readily to simultaneous studies in one patient of serum and cerebrospinal fluid levels, which are of great importance in the investigation of protein changes in neurologic disorders (19).

It was mainly for its possible application to cerebrospinal fluid as well as to serum, that it was thought desirable to investigate the published procedure of Jacox (20) for serum α-globulins. This investigator showed that, in high serum dilutions with collidine-sodium chloride buffer (pH 6.65), *only the α-globulins* reacted with the cationic detergent (Octab) to give a turbidity. Using a solution of Cohn's Fraction IV-1 as an α-globulin standard, he determined photometrically with a Lumetron colorimeter the α-globulin values of 26 sera and showed that the results obtained compared favorably with the electrophoretic method.

PRESENT STUDY

The present investigation deals with the application of this method to both serum and cerebrospinal fluid in which the turbidities due to the reaction of α-globulins with Octab are accurately measured by means of nephelometry, a procedure of greater sensitivity than colorimetry. The use of this instrument[1] required a number of modifications of the original Jacox (20) procedure. The modified method gave good checks for the α-globulin content of sera in comparison with the Tiselius moving-boundary electrophoretic procedure, and was then applied to the quantitative determination of the α-globulin levels of cerebrospinal fluids. The

[1] Coleman Photo-nephelometer, Model 7.

analyses were performed directly on these biologic fluids without prior separations and required only 0.50 ml. of fluid for each determination.

MATERIAL AND METHODS

Reagents and Apparatus

1. *Collidine buffer (.05M, pH 6.65)*: (a) Prepare a 0.2M solution of 2,4,6-trimethyl pyridine (Matheson No. 5479) by dissolving 2.64 ml. in 100 ml. of water solution. (b) To 25.0 ml. of solution (a) in a 100 ml. volumetric flask, add 42.0 ml. 0.1N HCl and 0.467 Gm. NaCl and dilute to mark with distilled water and mix. The *p*H of this solution should be checked with a glass electrode and adjusted to within 6.65 ± .05 *p*H units with 0.1N HCl or 0.2M collidine.

2. *Octab, 0.1%:* Dissolve 1.0 ml. Octab[2] in 99 ml. of distilled water and mix. Dilute this solution 1:10 with distilled water.

3. *Sodium chloride, .08M:* Dissolve 4.68 Gm. NaCl in 1 L. distilled water.

4. *α-globulin standard:* The α-globulin content (α_1 plus α_2) of pooled normal human sera was determined electrophoretically by means of multiple analyses with the Tiselius moving-boundary method in barbiturate buffer (*p*H 8.6), 0.1 ionic strength.

Procedure for the Determination of α-Globulins in Human Sera

Preparation of Sample

With an accurately calibrated micropipet, 0.10 ml. of serum is pipetted in duplicate into a tube (16 x 150 mm.). Then 15.0 ml. of .08M NaCl is added and the contents are mixed by inversion. Two 1.0-ml. aliquots from each sample of diluted serum (1:151) are pipetted into test tubes (15 x 100 mm.) and 8.0 ml. of the collidine buffer is added.

Nephelometer Setting

Before proceeding with the determination, the photonephelometer should be adjusted according to the instrument instructions for null point readings. The "36" nephelos standard is set to read 27 on the galvanometer scale. This increases the nephelos scale range from 0–130 to 0–170 units. This standard setting has to be checked from time to time during a series of readings to correct for minor fluctuations of the instrument.

[2] Octadecyl dimethyl benzyl ammonium chloride, Fairfield Laboratories, Plainfield, N. J. In place of Octab, cetyl dimethyl benzyl ammonium chloride may be used (Cetol); this is obtainable from Fine Organics, Inc., 211 E. 19th St., New York, N. Y. (personal communication from Dr. R. F. Jacox).

Determination of Unknown Samples

To the unknown tube is added 1.0 ml. of 0.1% Octab, and the contents are mixed by gentle inversion to avoid air bubbles, immediately transferred to a 19 x 105-mm. cuvet, and the galvanometer deflections are noted. As the reaction proceeds, the hairline indicator deflects to the right and is continually brought back to zero by means of the galvanometer balance knob until a *maximum* reading is obtained.

Determination of α-Globulin Serum Standard

A human serum standard, containing 1.08 Gm./100 ml. of α-globulins (α_1 plus α_2) as determined electrophoretically, is diluted and run nephelometrically, in duplicate, in exactly the same manner as described for the unknown samples.

Blank Determinations

Since serum is employed as the standard and high dilutions are used, blank values obtained for serum without Octab, or for Octab without serum, can be neglected. An exception to this is in the case of lipemic sera where the serum blank is significantly higher than the Octab blank and the difference between the two should be subtracted from the unknown nephelometric readings.

Calculations

$$\frac{\text{Maximum nephelometric reading (unknown)}}{\text{Maximum nephelometric reading (standard)}} \times \alpha\text{-Globulin standard (Gm./100 ml.)} = (\alpha_1 \text{ plus } \alpha_2) \text{ Globulins (Gm./100 ml. serum).}$$

Table 1. Comparison of Alpha ($\alpha_1 + \alpha_2$) Globulin Results Obtained with Nephelometric and Electrophoretic Methods for Normal and Pathologic Sera, and for Normal Cerebrospinal Fluid with Nephelometry

	$\alpha_1 + \alpha_2$ globulins (Gm./100 ml.)			
	Electrophoresis		*Nephelometry*	
	Mean ± S.D.	*Range*	*Mean ± S.D.*	*Range*
14 Normal sera	1.05 ± 0.12	0.94–1.27	1.13 ± 0.15	0.95–1.44
33 Pathologic sera	1.21 ± 0.21	0.71–1.64	1.22 ± 0.28	0.79–1.95
17 Cerebrospinal fluids (presumed normal)	..	..	8.56[a] ± 1.24	6.3[a] –10.2
	..	..	18.20[b] ± 2.16	14.8[b] –22.0

[a] Mg. α-globulins per 100 ml.
[b] Globulins/total protein × 100 (%).

The α-globulin content of 14 normal and of 33 randomly selected pathologic sera were determined in triplicate with the nephelometric procedure described above. The values obtained were compared with those found electrophoretically for the same samples with the Tiselius moving-boundary electrophoretic method as previously described (21) using a portable Aminco-Stern apparatus. The data obtained in these runs, including standard deviations, are given in Table 1.

Determination of α-Globulins in Cerebrospinal Fluid

Cerebrospinal fluid, 0.5 ml., is pipetted, in duplicate, into 15 x 100-mm. tubes. To each tube is added 0.50 ml. of a 1:151 dilution of the standard serum in .08M NaCl. Then 8.0 ml. of the collidine-NaCl buffer is added and the contents are mixed. The remainder of the procedure is exactly as described under *Procedure for the Determination of α-Globulins in Human Sera*. In addition, 0.50-, 0.75-, and 1.0-ml. aliquots of the diluted serum standard (1:151), prepared in duplicate, are also run in the same manner.

Calculations

Nephelometric reading (serum + C.S.F.) − Nephelometric reading (serum; 0.5 ml.) = Nephelometric reading (C.S.F.)

$$\frac{\text{Nephelometric reading (C.S.F.)}}{\text{Nephelometric reading (Standard Serum)}} \times \alpha\text{-Globulin standard (mg.)} \times \frac{100}{0.5}$$

$$= (\alpha_1 + \alpha_2)\text{-Globulins (mg/100 ml. C.S.F.).}$$

In these calculations, the nephelometric reading corresponding to the 0.50-ml., 0.75-ml., or 1.0-ml. standard value is used, dependent on which is closest to the unknown value, yields the most accurate results. Seventeen presumed normal cerebrospinal fluids were obtained from either psychotic individuals or from nonneurologic patients prior to spinal anesthesia. Their α-globulin values were determined by the method outlined and the results obtained are also given in Table 1.

RESULTS

Effect of Varying Concentrations of Reagents

According to the previously published results of Loomeijer (22) and Jacox (20), the α-globulin fraction of serum reacts with cationic detergents (e.g., Octab) in a lower *p*H range (6.5–7.5) than do any of the other protein fractions. However, Jacox (20, 23) showed that even at a

fixed *p*H, the turbidimetric readings obtained were markedly influenced by such factors as changes in the concentrations of buffer, detergent, and sodium chloride solutions used. The effect of these factors was re-investigated employing a standard serum of known α-globulin content diluted with collidine-NaCl buffer at *p*H 6.65. Since our conclusions for this system were in general the same as those of Jacox (20), they will only be briefly summarized here as follows:

(a) Variation of collidine-NaCl buffer from 0.025M to 0.06M had no appreciable effect on the nephelometric readings.
(b) Variation of final Octab concentration from 0.005% to 0.02% gave a gradual decrease in the nephelometric reading amounting to about 16 per cent between the lower and upper concentrations.
(c) Variation of sodium chloride concentration from 0.00 to 0.14M showed little change over the range from 0.05M to 0.10M NaCl.

The final concentrations of reagents used in these studies were arbitrarily chosen to conform to those used by Jacox (20, 23), since these correspond to the optimum conditions for serum where the change in nephelometric reading is least for variation in the amount of reagent used.

Assay of Cohn's Protein Fractions with Nephelometric Procedure

Jacox (20) has recommended the use of Cohn's Fraction IV-1 as a standard for the turbidimetric assay of serum α-globulin. This fraction, as well as other α-globulin-containing fractions (e.g., IV-4 and IV-6),

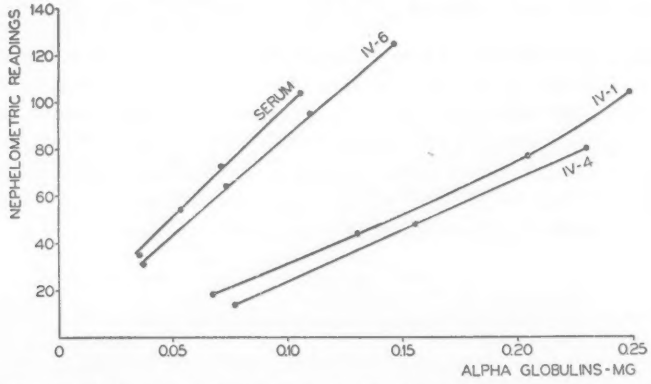

Fig. 1. Nephelometric readings obtained with the protein-detergent reaction at *p*H 6.65 with serum and Cohn's protein fractions for various concentrations (*mg.*) of *total* α-globulins.

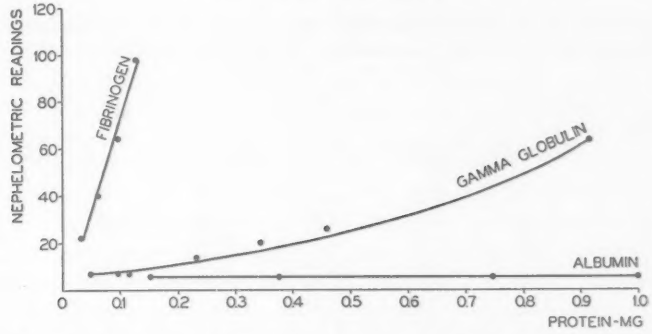

Fig. 2. Nephelometric readings obtained with the protein-detergent reaction at pH 6.65 for various non-α-containing protein fractions (Cohn Method 6[25]).

was investigated as possible standards for the nephelometric method in comparison with a standard serum of known α-globulin (electrophoretic) composition.

The results obtained in these runs are plotted as nephelometric reading versus milligrams of α-globulins and are shown in Fig. 1. In addition, a number of other protein fractions of known electrophoretic composition (24), as obtained by Cohn's Method 6(25), were run by the nephelometric procedure in order to determine their possible interferences with the α-globulin method. The protein fractions thus investigated include V (albumin—95%), II (γ-globulin—99%), and I (fibrinogen—67%). The results obtained in these experiments are shown in Fig. 2.

Since the results shown in Figs. 1 and 2 indicated that solutions of α-globulin-rich Fractions IV-1 and IV-6 might be useful as readily available standards in place of a serum standard for the nephelometric method, this possibility was investigated using six serum samples of known electrophoretic composition. The results obtained in these runs are given in Table 2.

Comparison of Turbidimetric and Nephelometric Methods for Serum α-globulins

The results obtained with the nephelometric procedure described above were compared with those determined with the previously published turbidimetric procedure of Jacox (20) for the same samples. The turbidities were read with both the Lumetron photoelectric colorimeter (402-E) as recommended by Jacox (20) as well as with the Beckman D.U. spectrophotometer at 440 mμ. In these runs both normal serum of known α-globulin content (1.08 Gm./100 ml.) and Fraction IV-1,

Table 2. Comparison of Electrophoretic Serum Alpha ($\alpha_1 + \alpha_2$) Globulin Values with Those Obtained Nephelometrically Using Human Serum or Fractions IV-6 or IV-1 as Standards

Sample #	*Total α-Globulins* (Gm./100 ml. serum)					
	Electrophoresis	*Human serum standard*	*Fraction IV-1 standard*	*Fraction IV-1 standard × 0.34*[a]	*Fraction IV-6 standard*	*Fraction IV-6 standard × 0.80*[a]
1	0.94	0.99	2.90	0.99	1.22	0.98
2	1.03	0.96	2.82	0.96	1.18	0.94
3	0.94	0.95	2.78	0.95	1.16	0.93
4	0.94	1.00	2.93	1.00	1.23	0.98
5	1.75	1.65	4.96	1.68	2.03	1.62
6	1.03	0.97	2.85	0.97	1.19	0.95
Mean	1.11	1.09	3.21	1.09	1.34	1.07

[a] See text, *Technical Considerations*, for explanation of experimental factors.

Table 3. Comparison of Serum Alpha ($\alpha_1 + \alpha_2$) Globulins Obtained by Electrophoretic, Nephelometric, and Turbidimetric (Jacox) Procedures Using Human Serum and Fraction IV-1 as Standards

Sample #	*Electrophoretic*	*Nephelometric*	*Turbidimetric*		
			Lumetron (402 E)		*Beckman D. U.*
		(Human serum standard)	*Human serum standard*	*Fraction IV-1 standard*	*(Fraction IV-1 standard)*
1	1.10	1.10	1.08	1.92	1.54
2	1.24	1.09	1.03	1.84	1.32
3	1.49	1.62	1.46	2.25	2.37
4	1.52	1.20	0.88	1.60	1.67
5	1.35	1.55	1.24	1.92	1.92
6	1.35	1.22	1.31	2.02	2.24
7	1.12	1.22	1.11	1.98	1.88
8	1.42	1.54	1.58	2.43	2.67
9	1.02	0.95	0.72	1.28	1.45
10	1.29	1.15	1.34	2.07	2.52
11	1.39	1.29	1.58	2.43	2.10
Mean ± S.D.	1.30 ± 0.16	1.27 ± 0.22	1.21 ± 0.28	1.98 ± 0.34	1.97 ± 0.45

as suggested by Jacox, were used as standards. The results of these runs are shown in Table 3 in comparison with the electrophoretic values for the same sera.

Discussion

Nephelometric Procedure

Nephelometric methods based on the photoelectric measurement of reflected light from white, dilute colloidal suspensions give a more stoichio-

metric relationship between concentration and readings than do turbidimetric procedures (26). In addition, the use of such an instrument as a null-reading device increases the sensitivity of the method two- to threefold (20, 27), which permits the accurate analysis of smaller amounts of substances—for instance, α-globulin content of cerebrospinal fluid.

While nephelometric analysis affords the investigator an increased order of sensitivity for many determinations, the production of uniform and stable suspensions requires careful attention to such factors as temperature, concentration of reagents, *p*H of system, presence of other salts, etc. It was by means of a careful investigation of such factors that Jacox (20, 23) was able to devise a procedure by which certain protein fractions, such as α-globulins, could be *quantitatively* determined, in the presence of the other protein fractions of serum, by means of their specific interaction with the cationic detergent (Octab).

Accuracy

The values obtained with the nephelometric method for the small series investigated are about 7 per cent higher on the average than are those found electrophoretically. However, the mean nephelometric value and standard deviation ($1.13 \pm .15$) checks closely with the mean electrophoretic α-globulin value previously reported for 140 normal individuals ($1.14 \pm .10$ Gm./100 ml.) (19). The mean values and standard deviations for the 33 pathologic sera given in Table 1 show excellent agreement between the two methods.

Analysis of the serum protein electrophoretic data for both normal and pathologic sera showed that correct *total* α-globulin values were obtained with the nephelometric method in spite of wide variations of either α_1- and/or α_2-globulins. However, in agreement with Jacox (20), an occasional disease serum gave a somewhat increased α-globulin value as compared to the electrophoretic results; for example, 1.73 as compared to 1.34 Gm./100 ml. Such discrepancies were found only in those cases where the electrophoretic α_2-globulin value was 2–3 times higher than the α_1-globulin result and was always accompanied by an elevated β-globulin level. From a clinical viewpoint, this is not a serious error as the electrophoretic total α-globulin values were above normal (1.20 Gm./100 ml.) in every case of this kind thus far encountered.

Effect of Lipoprotein and β-Fractions

The discrepancies discussed above furnish experimental proof that while the reaction of α-globulins with Octab, under the stated conditions,

is rather specific, it can be influenced by the presence of increased amounts of other protein fractions as well as by changes within the α-globulin fraction itself. In view of the fact that α_1-globulins contain a large proportion of lipoprotein substances (31), which are mainly concentrated in Cohn's Fraction IV-1, it was believed desirable to study the detergent interaction of this, as well as other α-globulin-containing fractions, with the nephelometric procedure. Such studies, in comparison with human serum, were of especial importance because of Jacox's recommended use of this fraction as a standard for his turbidimetric procedure.

The data obtained in these runs, expressed as *total* α-globulin concentration versus nephelometric readings, are shown graphically in Fig. 1. The results indicate that the amount of turbidity produced per unit of α-globulin concentration varies markedly for each fraction tested but is greatest for serum itself. For Cohn's fractions, the greatest amount of turbidity is given by Fraction IV-6, which is better than 95% electrophoretically-pure α_2 globulins whereas the IV-1 fraction which contains 77 per cent α_1-globulins plus 17 per cent α_2-globulins (electrophoretically) is only about one third as reactive. Fraction IV-4, which contains only 28 per cent α_2 globulins and 10 per cent α_1 globulins but 37 per cent β globulins gives nephelometric values approximately equal to those of Fraction IV-1, the lipoprotein-rich fractions. These findings would imply that the α-lipoproteins are not as reactive as are the α_1- or α_2-globulins and that the turbidities obtained for the isolated α-globulin-rich fractions may be influenced by the presence of other proteins—e.g., β globulins—such as exist in serum.

As a corollary to these experiments, a number of Cohn's other protein fractions—Fraction V (albumin—95%), Fraction II (γ-globulin—99%) and Fraction I (fibrinogen—67%)—were run with the procedure as described for the α-globulins. The nephelometric values obtained in these runs are plotted in Fig. 2 against the protein content (Kjeldahl) of each major fraction in milligrams. The data indicate that neither albumin nor γ-globulin react with Octab in amounts greatly in excess of those usually found in pathologic sera. Fibrinogen (Fraction I), however, does react to a considerable extent even when due allowance is made for the approximately 8 per cent of α_2-globulin, as was done in drawing the curve shown in Fig. 2. It has been assumed in evaluating the reactivity of the various Cohn protein fractions that in addition to albumin and γ-globulins, nonlipoprotein-containing β-globulin fractions *do not affect* the reaction with α-globulins. Some evidence for this is provided by the fact that Fraction IV-7, which is almost an electrophoretically

pure β_1-globulin of low molecular weight, does not react with Octab under the specified conditions.

Technical Considerations

The experimental data shown in Figs. 1 and 2 lead to the following tentative conclusions: Firstly, that the α-globulin fractions as separated with Cohn's Method 6(25) consist of mixtures of different proteins having similar electrophoretic mobilities as measured with the Tiselius apparatus. Some evidence for this is provided by the presence of easily denatured, high molecular weight α-lipoproteins (31) in some fractions, e.g., IV-1, which are not present in others, e.g., IV-4. Additional experimental evidence indicating the presence of a number of different α-globulins in human serum is provided by the work of Grabar and Williams (32, 33) who employed a combination of electrophoretic and agar gel diffusion technics to show the presence of 4–7 α-globulin fractions. Secondly, that on the basis of known α-globulin content, the greatest nephelometric values are obtained for serum. This, in itself, provides sufficient justification for its use as a standard in place of Cohn's protein Fraction IV-1 as previously recommended by Jacox (20). As is seen from the data in Table 2, except when a serum standard is used, the nephelometric and electrophoretic results differ by a constant experimental factor. Therefore, solutions of Cohn's Fraction IV-1 and IV-6 may be used as standards if such a factor is first obtained from the electrophoretic data. For this purpose, Fraction IV-6 has been found to be the closest to serum with respect to its reactivity with the detergent (Octab). No attempt was made to use Fraction IV-4 as a standard since the results obtained varied with time of contact of this fraction with the collidine-NaCl buffer.

Type of Measuring Instrument. Jacox (20) has emphasized that the Lumetron Model 402-E colorimeter with filter M 440 gave the steepest slope in terms of α-globulin concentration increments compared to a number of other photoelectric colorimeters tested. As our data in Table 3 show, essentially the same values were obtained for our samples whether this instrument or the Beckman D.U. spectrophotometer was used. Table 3 also demonstrates that a human serum standard of known α-globulin content gives the best checks with the electrophoretic values for the turbidimetric as well as for the nephelometric procedure.

It should, however, be emphasized that while the same protein-detergent interaction is being measured, the nature of turbidity measurements made with photoelectric colorimeters, or spectrophotometers, is

inherently different from those performed nephelometrically. The process of protein-detergent interaction consists of agglutination of the units of the soluble complex which in the presence of salt results in the formation of an increasing number of small light-scattering particles until a maximum is reached. This is followed by a decrease in the number of particles due to an increase in their size. The decrease in transmitted light intensity, as measured photoelectrically, is a resultant both of the blue light absorbed and reflected from the growing colloidal suspension in such a manner that the percentage transmission decreases continuously with time until a plateau is reached after about 20–30 minutes standing at room temperature. On the other hand, the nephelometer measures the light *reflected* at 90° to the incident beam, and this phenomenon is influenced to a great extent by the relative size and shape of the particles as well as their actual number, as is shown in Fig. 3. The time at which the maximum is reached decreases markedly with increasing concentration and at high concentrations occurs so rapidly

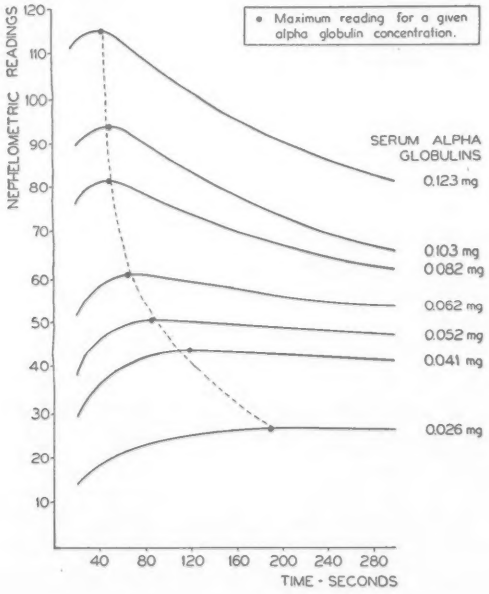

Fig. 3. Changes in the nephelometric values with time for various concentrations of serum α-globulins (*mg.*). Note the marked increase in *maximal* reading time with decreasing concentration.

as to make accurate detection of the end point difficult. A 1.0-ml. aliquot of 1:151 dilution of serum provides the best concentration for obtaining accurate, yet not unduly prolonged, end-point reading times of approximately 1 minute per determination. It should be emphasized that *only the maximum nephelometric readings* give a stoichiometric relationship with α-globulin concentration, as is illustrated by the data in Figs. 1 and 3.

Application to Cerebrospinal Fluid

Such data are not only of importance in obtaining reproducible and stoichiometric results for the α-globulin content of serum with the nephelometric method, but are of even greater importance when the procedure is being applied to the cerebrospinal fluid. Here it was found that a stoichiometric relationship between the volume of cerebrospinal fluid used and the nephelometric readings was not obtained unless serum was added to the system. This innovation had the additional advantage of permitting the use of smaller aliquots of cerebrospinal fluid—0.5 ml.—yet bringing the nephelometric readings into a range which gives the most accurate results.

Analysis of 17 presumably normal cerebrospinal fluids (see Table 1) with this method gave a mean total α-globulin value of 8.56 $\pm$ 1.24 mg./100 ml. and 18.20 $\pm$ 2.1% for the α-globulin/total protein ratio. These ratio values appear to be somewhat higher than those previously reported by other investigators, including Kabat and associates (16), who employed the moving-boundary method, and Schneider and Wallenius (17), Bücher *et al.* (34) and Roboz *et al.* (35), who employed filter-paper electrophoresis. However, it should be noted that investigators such as Schied and Schied (36) and Kabat *et al.* (16), who employed ultrafiltration through membranes as the technic for concentrating their protein samples, reported the complete absence of α-globulins in many of their fluids. Such findings are in disagreement with those of later investigators using substances of high molecular weight (Dextran, P.V.P., etc.) to remove water from dilute protein solutions contained in cellophane bags (17, 37).

The results of workers (34, 35) who concentrated the cerebrospinal-fluid proteins by means of prior precipitation with cold acetone followed by dissolution of the precipitate in minimum volumes of solution show even greater discrepancies from earlier ones. With these newer concentration technics substantial amounts of α-globulins were found in *all* cerebrospinal fluid samples including normals. Values obtained with the mem-

brane-dehydration methods average about 9.8% (17) as compared with 15.4% (34) and 17.1% (35), respectively, for the precipitation technics. It is evident that the latter values check more closely with those reported in this study with the direct nephelometric procedures—18.2 ± 2.2%.

As discussed by Bücher *et al.* (34), one reason for these marked differences in reported results is due to the known lability of the α-globulin fraction, and especially its lipoprotein portion, which is readily altered even under the mildest of conditions. Though there is evidence that the lipoproteins may travel at an entirely different rate than the corresponding globulins when conditions are altered, experimental evidence to support the former viewpoint is provided by the studies of Ewerbeck (38), who showed a 16 per cent loss of this fraction in concentrating a diluted serum of known electrophoretic composition. Additional evidence on the ease with which this fraction is lost is provided by work of Moore *et al.* (39), while more recent work on the concentration of gonadotrophins from urine by ultrafiltration (40) through collodion filters shows losses of 10–90 per cent of this protein by membrane adsorption.

It can be concluded from the aforementioned experimental data that the most important consideration for obtaining accurate cerebrospinal fluid α-globulin values is to avoid losses due to concentrating dilute protein solutions. It would then be expected that direct analysis of this fluid would give the somewhat higher results obtained with the detergent-nephelometric procedure. Based on the interaction of fibrinogen with Octab (Fig. 2) under the same experimental conditions as for α-globulins, the possibility still exists that the 1–3 per cent higher results are due to traces of this component too small to be detected through its clotting properties (40). However, Kabat *et al.* (16) have shown that this fraction is usually present only in cerebrospinal fluids with total proteins greater than 100 mg./100 ml. and that even in these pathologic fluids it remains at about 5 per cent of the total protein. In addition, the presence of this fraction has not been reported by investigators employing paper electrophoretic technics (17, 34, 35, 37), even in pathologic cerebrospinal fluids, although the presence of a new fraction (7) which moves closer to the β-globulin than to the γ-globulins has been reported by Bücher *et al.* (34). This problem, as well as the development of a complete system of direct chemical cerebrospinal-fluid protein-fraction analysis is presently under investigation.

DISCUSSION

Synthetic detergents may be classified as nonionic, anionic, and cationic, according to the sign of the charge on the hydrocarbon (hydro-

phobic) group after dissociation of the ionic (hydrophilic) group in solution. Only ionic detergents have been found to precipitate proteins from soultion and then in a *p*H region where the protein carries a charge of opposite sign to that of the detergent. In the case of mixtures it is essential that the reaction be carried out in a buffered *p*H region where its solubility is minimal and where there is the least likelihood of contamination from other protein fractions present. This is essentially the experimental situation described in this paper as concerns the interaction of the serum α-globulins, which carry a negative charge at *p*H 6.65, with the positively charged cationic detergent, Octab.

Factors in Protein-Detergent Interaction

As previously mentioned, the quantitative aspects of protein-detergent interactions are not only governed by the *p*H of the medium and the corresponding sign and magnitude of charge of the reactants, but also by such factors as the protein/detergent weight ratio, the temperature, and the ionic strength (or salt content) of the solution (41). The character of the hydrocarbon chain of the cationic detergent employed in the reaction is also of some importance, as Jacox (20) found that greater turbidity per unit of protein is obtained for a dimethyl-benzyl-substituted C_{18}-hydrocarbon chain than for a trimethyl- or ethyl-substituted one. The degree of turbidity is also a function of the chain length, being greatest for about a C_{16}-chain. It was such information which led Putnam (42) to suggest that although protein-detergent combination may involve primarily electrostatic forces, the complex is stabilized by factors or specific affinity (Van der Waals forces) between the nonpolar groups of the bound detergent ions.

Protein/Detergent Ratio

In their studies of the precipitation of horse serum albumin at *p*H 4.5 with the anionic detergent, sodium dodecyl sulfate, Putnam and Neurath (41) reported an optimal range of protein/detergent weight ratio at which 100% of the protein precipitated, and outside this range there was incomplete or no precipitation. They also found that the width of the range at which 100% precipitation occurs varies directly with the protein concentration. This means that the protein/detergent ratio becomes a less critical factor at higher protein concentrations.

In the present study, a slight decline—about 16 per cent—was observed in the nephelometric readings with a fourfold increase in the protein/detergent ratio. This would imply that while under the given experimental conditions the α-globulins form insoluble complexes with

Octab, some of the detergent may also be bound to the other serum protein fractions in the form of soluble complexes. This phenomenon would then serve to explain the experimental fact that variation of the protein/detergent ratio is of minor importance for serum determinations where stoichiometric results are obtained over a wide range of α-globulin values, but has greater significance in the case of cerebrospinal fluids where low combining ratios may result in a lack of stoichiometry. In the latter case it was found that the proper protein/detergent combining ratio, and consequently stoichiometric results, could be readily achieved by means of the addition of a serum standard of known α-globulin content to the unknown cerebrospinal fluid sample prior to performing the nephelometric determination.

Temperature

Putnam and Neurath (41) have studied the effect of temperature on protein/detergent interactions and have found little change in the region of maximal precipitation. While this factor was not checked experimentally in this paper, no significant changes were noted in the serum α-globulin standard values over a considerable range of room temperature during the past year.

Noninterference of Albumin

A rather surprising finding which warrants additional comment is the inability of albumin, which carries a large negative charge at *p*H 6.65, to form insoluble complexes with the positively charged detergent (Octab). In a recent review article, Klotz (44) states that among ions of equal size organic cations are bound much less than anions by serum albumin and that this may indicate that the -COO^- chains in proteins are blocked, perhaps by interaction with -OH groups from hydroxy amino acid residues. At any rate, as pointed out by Glassman (45) and subsequently by Jacox (20), albumin must carry a substantial *negative* charge before precipitation with a cationic detergent occurs. It is this unique property of albumin that makes the sensitive Octab reaction relatively specific for α-globulins, in both serum and cerebrospinal fluid, with little interference from the other protein fractions present.

Ionic Strength

Another important experimental factor which aids in the specificity of the cationic detergent (Octab) reaction for α-globulins is the effect of salt concentration (or the ionic strength) of the system. In agreement with Jacox (20, 23), it was found that little or no precipitation of the

complex occurs in the absence of salt and that with increasing salt concentration the nephelometric readings approach an asymptotic value. Since, except for albumin, the α-globulins carry the largest negative charge, the electrostatic bonds formed with Octab should result in the most electrically neutral stable complex. The addition of salt would then provide an ionic atmosphere of opposite charge and sufficient strength to reduce any excess charge on the complex to a point where agglutination and precipitation of the charged particles can take place. Other soluble protein-detergent complexes carrying larger charges will not have their charges as readily neutralized by salt concentration and will remain in solution. This is analogous to the reaction mechanism by which turbidities or precipitates of certain protein fractions evolve in protein flocculation reactions (46). The aforegoing discussion was confined to those aspects of protein-detergent interaction which have some bearing on our experimental results. Excellent review articles dealing with the mechanism of these reactions, their effects in biological systems, etc., have been published by Putnam (41–43).

Clinical Applications and Significance of Quantitative α-Globulin Determination

Quantitative changes of the α-globulin protein fractions, in either serum or cerebrospinal fluid, are in general nonspecific in that they occur in many different clinical conditions. They may, however, be of some value in either establishing the presence of an active disease process or in helping to rule out or confirm one of several clinical diagnoses. Increases in serum α-globulin levels, usually associated with decreased albumin values, have been found in such diseases as nephrosis, cirrhosis, pneumonia, acute rheumatic fever, typhus, etc. and generally in conditions involving fevers or considerable inflammation or tissue destruction, e.g., carcinomas or sarcomas (47). Other recent review articles which give data pertaining to disease states where abnormal α_1- or α_2-globulin values have been reported are those by Leutscher (48), Ardry (49), Flynn (50), and Fisher (15).

Articles dealing with the quantitative analysis of the cerebrospinal-fluid protein fractions of both normal and pathologic fluids are assuming increasing importance in the literature (16, 17, 34–38). While the α-globulin values obtained by most investigators must be accepted with some caution because of possible losses during the concentration step, the errors involved would tend to lower the results obtained. Therefore, the increased cerebrospinal fluid α-globulin values found in such diseases as meningitis, syphilis, mumps, tuberculosis, poliomyelitis, etc. (51), would be even higher with our procedure than those reported by other

investigators. The extension of the nephelometric procedure to the quantitative analysis of the α-globulin content of cerebrospinal fluid from patients with a wide variety of disorders is presently under investigation. Special attention is being directed toward the investigation of protein changes in diseases of the central nervous system, since, as previously mentioned, synthesis of proteins may occur in the meningeal space in such conditions without corresponding increases in the serum proteins (16, 52). Preliminary data obtained in such studies indicate that significant elevations of the cerebrospinal fluid α-globulins, and of the γ-globulins as well (19, 53), occurs in such neurologic conditions as multiple sclerosis, hemiplegias, and the like.

SUMMARY

1. A micronephelometric method is presented for the quantitative determination of total α-globulins in both serum and cerebrospinal fluid. The method is based on the relatively specific interaction of this protein fraction with the cationic detergent, Octab, at *p*H 6.65 in collidine-NaCl (.08M) buffer.

2. The various Cohn's protein fractions (Method 6) (25) were investigated with the nephelometric procedure. In comparison with normal serum, Fractions IV-6, IV-1, and IV-4, respectively showed the greatest detergent reactivity per milligram of α-globulin.

3. The variations of such factors as the concentrations of reagents, protein/detergent ratio, and salt concentration were reinvestigated.

4. Analysis of 14 normal and 33 pathologic sera gave excellent checks with the *total* α-globulin values obtained with the Tiselius electrophoretic method.

5. Application of the procedure to the analysis of the α-globulin content of cerebrospinal fluid required the addition of standard serum to 0.5-ml. aliquots of spinal fluid for stoichiometric results.

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ABSTRACTS

Porphobilinogen and δ-amino levulinic acid in acute porphyria. S. Granick and H. G. Vanden Schrieck (*Rockefeller Institute of Medical Research, New York, N. Y.*)

The porphobilinogen excreted in the urine of a patient with a high excretion was determined with Ehrlich's reagent to be 80–100 mg./day. Delta-amino levulinic acid, which has not been reported previously in the urine of acute porphyria patients, was present in amounts of about 40 mg./day. For its determination porphobilinogen was removed from the urine by passage through a column of Dowex-2 × 2 resin as the acetate. The δ-amino levulinic acid was adsorbed from the eluate on Dowex-50 × 8 resin as the chloride. It was eluted with 0.5M phosphate buffer, *p*H 7.0, and coupled with acetyl acetone at *p*H 4.6 by heating the mixture in a boiling water bath for 10 minutes. A pyrrole compound formed which gave a color with an equal volume of Ehrlich's reagent. This color was read at 555 mμ 5 minutes after mixing. This compound apparently produced no symptoms when injected into a rat but was converted to porphyrins. It was also converted to porphyrins by a suspension of guinea-pig-liver cells. If in acute porphyria there is a block in a metabolic step above the formation of porphobilinogen in the liver, the data from this case indicate that the normal human liver may synthesize more than 100 mg. of heme a day, which may be converted to bile pigment.—*Proc. Soc. Exp. Biol. Med.* **88,** 270 (1955). (M. K.)

Xanthurenic acid excretion studies in diabetics with and without retinopathy. D. A. Rosen, G. D. Maengwyn-Davies, B. Becker, H. H. Stone, and J. S. Friedenwald (*Johns Hopkins Hospital and University, Baltimore, Md.*)

Diabetic and nondiabetic subjects were given an oral test dose of 10 Gm. of *dl*-tryptophane, and the xanthurenic acid excreted in the urine collected during the following 24 hours was determined by the method of Wachstein and Gudaitis [*Am. J. Clin. Pathol.* **22,** 652 (1952)]. The mean excretion of 8 nondiabetics was 9.76 mg., of 8 diabetics without retinopathy 25.02 mg., and of 12 diabetics with retinopathy 38.22 mg. The difference between the two groups of diabetics was not statistically significant. In the 6 subjects with the highest excretions, injections of 300 mg. of pyridoxine caused a marked drop in xanthurenic acid excretion.—*Proc. Soc. Exp. Biol. Med.* **88,** 321 (1955).

(M. K.)

Plasma glutamine and oxypurine content in patients with gout. S. Segal and J. B. Wyngaarden (*National Institutes of Health, Bethesda, Md.*)

Plasma glutamine levels were determined in 10 normal and 10 gouty subjects by means of a specific enzymatic hydrolysis and also after acid hydrolysis followed by measurement of the nitrogen liberated. Uric acid and oxypurines were also estimated. No differences were observed between the two groups in their plasma glutamine content, and the plasma oxypurine was definitely elevated in only 1 gouty subject. This study failed to confirm previous reports of low levels of glutamine and of markedly high levels of oxypurines in the plasma of patients with gout.—*Proc. Soc. Exp. Biol. Med.* **88,** 342 (1955). (M. K.)

Ammonia levels in blood and cerebrospinal fluid. W. V. McDermott, Jr., R. D. Adams, and A. G. Riddell (*Harvard Medical School and Massachusetts General Hospital, Boston, Mass.*)

Simultaneous determinations were made of the ammonia in peripheral blood and in cerebrospinal fluid by a modification of the microdiffusion technic of Conway [*J. Clin. Invest.* **33,** 1 (1954)]. In 6 patients undergoing surgery who had no liver disease and who had blood ammonia-nitrogen levels of 51 to 65 μg./100 ml., there was no appreciable ammonia in the cerebrospinal fluid. In 22 patients with liver diseases under similar conditions there was a linear relationship between the blood and spinal fluid ammonia values, with the latter figures always somewhat lower. Five cases with spinal fluid values above 105 μg./100 ml. had disturbances of the central nervous system.—*Proc. Soc. Exp. Biol. Med.* **88,** 380 (1955). (M. K.)

A rapid procedure for the determination of lead in blood or urine in the presence of organic chelating compounds. S. P. Bessman and E. C. Layne, Jr. (*Children's Hospital, Washington, D. C.*)

The present method, a modification of the dithizone-colorimetric method [*Ind. Eng. Chem., Anal. Ed.* **14,** 904 (1942)], is designed to shorten the time required for analysis, to lessen the danger of contamination, and to diminish the large quantity of blood or urine usually required for analysis.

The digestion and extraction are carried out in a single glass-stoppered digestion tube (diagram given) so that quantitative transfers of the test solutions at any point before the excess dithizone has been removed are eliminated. Resin deionized water eliminates the need for a glass distillation apparatus.

Only 2 ml. of blood or 10 ml. of urine are required for analysis. Twelve determinations of blood from patients with no history of exposure to lead gave a mean of 18.2 ± 11 μg./100 ml., with a range of 0 to 32 μg. Calcium ethylene diamine tetraäcetate at levels equivalent to 500 mg./100 ml. in urine and 2500 mg./100 ml. in blood does not interfere.—*J. Lab. Clin. Med.* **45,** 159 (1955). (G. D.)

The direct microdetermination of calcium in the urine by nephelometry. G. S. Stewart, H. F. Bowen, and E. M. Cullerton (*University of Illinois College of Medicine, Chicago, Ill.*)

A micromethod for the direct nephelometric determination of calcium in urine is described in which calcium is estimated as a stabilized colloidal suspension of the oleate in ammoniacal solution. The method is comparable in precision to those involving ashing and oxalate precipitation and requires a maximum of 0.1 ml. of urine.

Complete solution of insoluble calcium salts is insured by shaking the total urine sample with HCl (1 ml. conc. HCl/100 ml. of urine). A small portion of the sample is filtered and 0.1 ml. of the filtered specimen is added to 4.5 ml. of H_2O in a cuvette. Three drops of 37% NH_4OH is added, followed by 0.4 ml. of Duponol-oleate reagent, and the solution is thoroughly mixed. One-half hour later the sample is read against a reagent blank. Standards containing 10 μg. of Ca are similarly treated. The nephelometer is used as a null point instrument.

The following reagents are required:

1. Potassium oleate: 16.5 ml. of oleic acid are shaken with 10 ml. of water containing 3.2 Gm. of KOH. The emulsion is transferred to a flask with 100 ml. of 70% ethyl alcohol, refluxed 1 hour, and diluted to 500 ml. with water.
2. Sodium lauryl sulfate: 10% aqueous solution of Duponol.
3. Duponol-oleate reagent: 5 parts of potassium oleate reagent are mixed with 2 parts of 10% Duponol.

The solution is stable but must be cleared by filtration.—*J. Lab. Clin. Med.* **45,** 653 (1955). (G. D.)

A comparison of three methods, utilizing different principles, for the determination of uric acid in biological fluids. P. Lous and O. Sylvest (*Sundby Hospital, Copenhagen, Denmark*)

The authors determined the uric acid concentration of 200 samples of serum by 3 different procedures—the colorimetric method of Folin [*J. Biol. Chem.* **13,** 469 (1912/13)], the titrimetric method of Brøchner-Mortensen (*Acta med. scandinav., Supp.* **84,** 1937) and the uricase method of Praetorious [*Scand. J. Clin. & Lab. Invest.* **1,** 222 (1949)].

No correlation between the results could be demonstrated. In most instances the colorimetric procedure gave a lower result and the titrimetric procedure a higher result than that obtained with the uricase method. Since the uricase method is specific for uric acid while the other procedures are not, it is recommended that the uricase method be used. Its only objection is that it requires a rather expensive instrument, since the absorption is measured in the ultraviolet range.—*Scand. J. Clin. & Lab. Invest.* **6,** 40 (1954). (E. V.)

Glucose and acetone as sources of error in plasma "creatinine" determinations. N. H. Haugen (*Ullevål Hospital, Oslo, Norway*)

In diabetics with elevated blood sugar levels and ketonemia very high plasma "creatinine" levels are found by the Jaffe creatinine procedure, even when the

color is read 10 minutes after the addition of the reagents. If the color is not read until 40 minutes after the addition of the reagents, the increase is even more marked. The falsely elevated values are due to high glucose and acetone levels and perhaps to other substances such as ketone bodies. Most of these "pseudocreatinine" compounds can be removed with Lloyd's reagent.—*Scand. J. Lab. & Clin. Invest.* **6,** 17 (1954). (E. V.)

The estimation of Evans blue in plasma. G. A. Bedwell, J. Patterson, and J. Swale (*Charing Cross Hospital and Charing Cross Hospital Medical School, London, England*)

Evans blue is isolated from plasma by chromatography, using 50 mg. of amorphous cellulose for the adsorption of the dye. The dye is then eluted from the column with acetone. A funnel heater jacket surrounds the column and the adsorption and elution are carried out at a temperature of 45° to obtain complete recovery of the dye.

With this method errors due to hemolyzed or lipemic plasma are eliminated. A 4 ml. sample of plasma is used and the entire procedure takes about 1 hour to complete.—*J. Clin. Pathol.* **8,** 61 (1955). (E. V.)

Adrenocortical activity in the preoperative period. C. Franksson and C. A. Gemzell (*Karolinska Hospital, Stockholm, Sweden*).

The adrenocortical activity of 33 surgical patients in the preoperative period was evaluated by the determination of 17-hydroxycorticosteroid levels in peripheral blood. Twenty-six of the 33 patients showed an increase in the concentration of plasma steroids. It is suggested that this is due to psychic tension.—*J. Clin. Endocrinol. and Metabolism* **15,** 1069 (1955). (M. R.)

Clinical application of the simplified Silber-Porter method for determining plasma 17-hydroxycorticosteroids. E. Z. Wallace, N. P. Christy, and J. W. Jailer (*College of Physicians and Surgeons, Columbia University, New York, N. Y.*).

The Silber-Porter technic [*J. Biol. Chem.* **210,** 923 (1954)] was modified slightly for the measurement of plasma 17,21-dihydroxy-20-ketosteroids. The range of values in 50 normal subjects was 4 to 32 μg. per 100 ml. of plasma, with an average of 16 ± 6.9 μg. Normal plasma levels were observed in most patients with nonendocrine disease and in those with disorders of glands other than the adrenal cortex. Elevated values were found in diabetic acidosis, in 3 patients with renal disease, and in 2 moribund patients, none of whom showed hyperadrenalism. Patients with renal insufficiency had normal or elevated plasma corticoid levels, in contrast to the abnormally low urinary 17-ketosteroid and corticoid values sometimes seen.

The method was especially useful in the diagnosis of hyperadrenalism; cases of Cushing's syndrome showed a range of 29 to 107 μg. per 100 ml. Low values were found in primary adrenal insufficiency and in hypoadrenalism secondary to pituitary insufficiency.—*J. Clin. Endocrinol. and Metabolism* **15,** 1073 (1955). (M. R.)

A simplified dye method for estimating plasma volume. H. A. Frank and M. H. Carr (*University of Kansas School of Medicine, Kansas City, Mo.*).

A simplified method for estimating plasma volume with Evans blue dye is presented. The procedure is performed on aliquots of diluted dye, eliminating entirely the need to determine absolute amounts of dye. Problems due to differing optical densities of T-1824 in different solvents are eliminated by preparing all samples in a solvent of constant composition, using the patient's own serum. If recognizable hemolysis is present in any sample, a correction is made by reading the optical density at 540 mμ as well as at 620 mμ and applying a correction formula.—*J. Lab. Clin. Med.* **45,** 977 (1955). (G. J. D.)

A method for the determination of acetone in expired air—with special reference to diabetic ketosis. R. Dybkaer and O. Sylvest (*Sundby Hospital and De Gamles By, Copenhagen, Denmark*)

Expired air is bubbled for 1–2 minutes through 2 ml. of 0.2% 2,4-dinitrophenylhydrazine in 2N HCl. The acetone-dinitrophenylhydrazine thus developed is extracted with 2 ml. of carbon tetrachloride. Yellow coloring of the latter indicates acetone. The method is sensitive to 0.008–0.010 mg. of acetone. Expired air from normal individuals gives no positive reaction. The only compound which may be confused with acetone is acetaldehyde which is found in the expired air from patients after ingestion of alcohol.

The procedure is more sensitive than procedures for the determination of ketones in urine and is particularly advantageous in cases of diabetic coma.—*Scand. J. Clin. & Lab. Invest.* **6,** 311 (1954). (E. V.)

Depression of serum alkaline phosphatase activity by human serum albumin. P. H. Henneman, G. M. Rourke, and W. P. U. Jackson (*Massachusetts General Hospital and Harvard Medical School, Boston, Mass.*)

Experiments performed in vitro show that human albumin in high concentration can cause a reduction of serum alkaline phosphatase activity. In vivo experiments show that with daily dosages of 50 Gm. of albumin the alkaline phosphatase activity may be reduced as much as 50 per cent. Indications are that slightly elevated serum alkaline phosphatase levels may sometimes be explained on the basis of hypoalbuminemia alone.—*J. Biol. Chem.* **213,** 19 (1955). (J. A.)

The determination of total lipids in blood serum. W. M. Sperry and F. C. Brand (*New York State Psychiatric Institute and College of Physicians and Surgeons, Columbia University, New York, N. Y.*)

Total lipids are extracted with boiling methyl alcohol and chloroform. The extract is purified by a water diffusion procedure requiring meticulous technic. Methanol is added and the mixture is evaporated to dryness in vacuo in an atmosphere of nitrogen. The residue is redissolved, filtered, dried and weighed. Recoveries and comparisons with other accepted methods were within 1 per cent.—*J. Biol. Chem.* **213,** 69 (1955). (J. A.)

The application of the ceric sulphate-arsenious acid reaction to the detection of thyroxine and related substances. C. H. Bowden, N. F. MacLagan and J. H. Wilkinson (*Westminster Medical School, London, England*)

Inorganic and organic iodine-containing compounds were detected in solution and on paper chromatograms by using the ceric sulphate-arsenious acid reaction. As little as 0.01 μg. of potassium iodide and 0.1 μg. of thyroxine are detectable upon chromatograms but 20 times these amounts are necessary for detection in solution.—*Biochem. J.* **59,** 93 (1955). (A. K.)

A modified Conway unit for microdiffusion analysis. K. J. Obrink (*University of Uppsala, Sweden*)

The ordinary Conway unit has been modified by the addition of a peripheral chamber, to be filled with the same liquid as the outer diffusion chamber (except the fluid to be analyzed). An inverted Petri dish dipped into this chamber forms a liquid trap. This eliminates the use of a fixative for sealing the unit.—*Biochem. J.* **59,** 134 (1955). (A. K.)

An atypical electrophoretic peak in serum of patients with familial primary systemic amyloidosis. W. D. Block, J. G. Rukavina, and A. C. Curtis (*University of Michigan, Ann Arbor, Mich.*).

Data are presented from moving-boundary electrophoretic analyses of the sera from 5 members of a family with primary systemic amyloidosis. The values for total protein were normal with a tendency for the albumin to be somewhat low and the globulin slightly elevated. The α_1, β, and γ-globulins were normal, with a slightly decreased α_2-globulin. However, in all the patients an abnormal peak was seen between the α_2 and β-globulins, which has been designated α_2^1.—*Proc. Soc. Exp. Biol. Med.* **89,** 175 (1955). (M. M. K.)

Paper electrophoresis as a quantitative method. W. P. Jencks, M. R. Jetton, and E. L. Durrum (*Army Medical Service Graduate School, Washington, D. C.*).

The quantity of bromophenol blue dye bound by denatured proteins on filter paper is dependent on the time of heat denaturation of the protein, the time of staining, and the amount of rinsing. These times vary with different proteins. Bromophenol blue on paper does not follow Beer's law when directly scanned. When normal serum is stained under the conditions described, the deviation requires correction for only the densely stained albumin fraction. A staining procedure is described which gives a linear relationship between protein and dye concentration.—*Biochem. J.* **60,** 205 (1955). (A. K.)

Preliminary investigations into the nature of neutral 17-ketosteroids in human plasma. G. W. Clayton, A. M. Bongiovanni, and C. Papadatos (*Johns Hopkins University Medical School, Baltimore, Md.*).

A pool of 950 ml. of plasma from 4 healthy adult males was examined for neutral 17-ketosteroid content. The ketosteroids were extracted with ether-ethanol and

the conjugates hydrolyzed and purified by various solvent partitions and Girard separation. The crude neutral fraction was chromatographed on alumina. Dehydroepiandrosterone (65 per cent of the total neutral ketonic fraction) and androsterone (11 per cent of the total neutral ketonic fraction) were identified. Trace amounts of unidentified 17-ketosteroids were found. A possible explanation is that a greater proportion of dehydroepiandrosterone is found in the plasma since it is firmly bound to the protein and less easily excreted. Small amounts of highly polar Zimmermann-reacting material probably represent 11-oxygenated 17-ketosteroids or 20-ketones.—*J. Clin. Endocrinol. and Metabolism* **15,** 693 (1955). (M. R.)

Determination of uric acid in serum by a carbonate method. W. T. Caraway (*Rhode Island Hospital, Providence, R. I.*).

A simple reproducible method is described for the determination of uric acid in serum. Two stable reagents, sodium carbonate and dilute phosphotungstic acid, are added to a protein-free tungstic acid filtrate of serum to develop a color which is measured photometrically. No cyanide is used. In replicate analyses the reproducibility of the carbonate method was found to be much superior to that of the cyanide method.

Serum proteins are precipitated by adding 9.0 ml. of precipitating reagent to 1.0 ml. of serum. After centrifugation or filtration, 5.0 ml. of clear filtrate are transferred to a test tube. A blank tube is prepared by substituting 5.0 ml. of water for the filtrate. To each tube is added 1 ml. of 10% sodium carbonate, and the solutions are mixed by swirling, after which 1 ml. of dilute phosphotungstic acid is added to each tube and the solutions are mixed at once. The optical density is determined 30 to 50 minutes later at a wavelength of 700 mμ. The concentration of uric acid is obtained from a calibration curve. Recoveries of uric acid added to serum varied from 85 to 95 per cent.

The reagents are prepared as follows:

1. *Stock phosphotungstic acid:* Dissolve 100 Gm. of molybdate-free sodium tungstate in 800 ml. of water in a Florence flask. Add 80 ml. of 85% phosphoric acid. Reflux gently for 2 hours. Cool and dilute to 1 L. with water. Stable indefinitely in a dark bottle.

2. *Dilute phosphotungstic acid:* Dilute 10 ml. of stock to 100 ml. with water. Stable when stored in a brown bottle.

3. 10% sodium carbonate.

4. *Stock uric acid standard, 0.2 mg./ml.:* Transfer 100 mg. of pure uric acid to a 500-ml. volumetric flask. Dissolve separately 2.30 Gm. anhydrous Na_2HPO_4 in 300 ml. of warm water, transfer to the volumetric flask and mix until the uric acid is completely dissolved. Cool and add 0.9 ml. of glacial acetic acid. Dilute to volume with water.

5. *Uric acid working standards:* Aliquots of the stock standard are prepared to provide solutions covering the range 0.1 to 1.6 mg. uric acid/100 ml. These solutions are prepared fresh on the day used.—*Am. J. Clin. Pathol.* **25,** 840 (1955). (H. E. T.)

A clinical method for determination of plasma galactose in tolerance tests. N. Tygstrup, K. Winkler, E. Lund, and H. C. Engell (*University Medical Clinic B, Rigshospitalet, Copenhagen, Denmark*)

A method is described for determining galactose in plasma. Glucose is fermented with a specific glucose oxidase, Notatin, which is an improvement over the use of yeast. Proteins are precipitated with ferric sulfate and barium carbonate [Steiner, *J. Biol. Chem.* **98,** 289 (1932)]. The nonfermentable reducing substance is determined colorimetrically using Somogyi's modified copper reagent [*J. Biol. Chem.* **195,** 19 (1952)] and Nelson's arsenic reagent [*J. Biol. Chem.* **153,** 375 (1944)]. The standard deviation of the method was found to be about 2 mg./100 ml. at concentrations of 100 mg./100 ml. of galactose.—*Scand. J. Lab. & Clin. Invest.* **6,** 43 (1954). (E. V.)

Phosphate contamination of commercial heparin. M. G. McGeown, E. Martin, and D. W. Neill (*Queen's University and Royal Victoria Hospital, Belfast, Ireland*).

In the course of routine estimations of plasma inorganic phosphorus, it was found that the heparin used was heavily contaminated with phosphors and was responsible for erroneously high values.—*J. Clin. Pathol.* **8,** 247 (1955). (E. V.)

Colour production and stability in the Folin and Wu method of blood glucose estimation. S. Dische (*Royal Air Force Institute of Pathology and Tropical Medicine, Halton, Aylesbury, Buckinghamshire, England.*)

A new modification of the original Folin and Wu method is described which eliminates the fading of the final color and also simplifies the technic. A phosphomolybdic working reagent is prepared by mixing 150 ml. of the reagent of Folin and Wu [*J. Biol. Chem.* **41,** 367 (1920)] with 25 ml. of phosphoric acid and 325 ml. of distilled water. After the customary heating with alkaline copper reagent for 6 minutes in a boiling water bath, 10 ml. of the dilute phosphomolybdic reagent are added immediately. After 1 minute the contents of the tube are mixed and read in a photoelectric colorimeter, using a red filter.—*J. Clin. Pathol.* **8,** 253 (1955). (E. V.)

The determination of calcium in biologic material. W. H. Horner (*Army Medical Service Graduate School, Walter Reed Army Medical Center, Washington, D. C.*).

A simple, rapid, accurate photometric titration method for the determination of calcium in serum, urine, and ashed biologic specimens is described, using disodium dihydrogen ethylenediamine tetraäcetate (EDTA). The principle of "internally blanking" the photometric titration is employed to eliminate the titration of a blank. A detailed diagram of an automatic titrating assembly employing intermittent air agitation as a means of mixing is also included in the article.—*J. Lab. Clin. Med.* **45,** 951 (1955). (G. J. D.)

An efficient clinical method for determining the CO_2 content of serum. A. Kahn (*University of Chicago, Chicago, Ill.*).

Using a Coleman *p*H meter adapted for measuring *p*H under anaerobic conditions with an accuracy of ± 0.01 *p*H units, the CO_2 content of serum can be determined simply and rapidly. The *p*H of 1 ml. of serum is measured before and after the sample has been equilibrated with a gas mixture of known CO_2 content. From the change in *p*H the CO_2 content can be calculated or read from a nomogram. The electrode chamber should be held at a constant temperature of 37°. The gas mixture for equilibrating contains 5–6 % CO_2 and 94–95 % N_2; the exact composition is determined using a Haldane or Van Slyke manometric apparatus. The CO_2 values obtained by this method compare favorably with those obtained by the Van Slyke manometric procedure.—*J. Lab. Clin. Med.* **46,** 312 (1955). (G. J. D.)

Manual of workshop in clinical hemoglobinometry of American Society of Clinical Pathologists (Condensed Version). F. W. Sunderman, B. E. Copeland, R. P. MacFate, V. E. Martens, H. N. Naumann, and G. F. Stevenson.

Technics are given in detail for the gravimetric calibration of 20-cu. mm. pipets, the determination of iron content of blood, oxyhemoglobin measurements, and the identification of hemoglobin derivatives.—*Am. J. Clin. Pathol.* **25,** 695 (1955). (E. V.)

Copper sulfate as reagent in cerebrospinal fluid analysis. C. de Chenar (*Austin State Hospital, Austin, Texas*).

Copper sulfate reagent provides a globulin reaction of the cerebrospinal fluid sensitive enough to be regarded as an approximate quantitative test. The reagent is a 0.05 % solution of reagent-grade $CuSO_4 \cdot 5H_2O$ in triple-distilled water. One-half milliter of this reagent is pipetted into 3 small test tubes, and 0.25 ml. of the spinal fluid is added to the first tube, 0.5 ml. to the second, and 0.75 ml. to the third. When no pathologic alteration is present, an opalescence is seen in the third tube. In cases of pathologic change associated with increased globulin, opalescence or precipitation takes place in the second and first tubes, depending on the amount of globulin, particularly gamma globulin, in the spinal fluid. The reaction was positive in several cases where other tests for globulin did not give a definite reaction.—*Texas Repts. Biol. Med.* **12,** 453 (1954). (E. V.)

A study of methods for the prediction of plasma volume. S. R. Inkley, L. Brooks, and H. Krieger (*University of Cleveland and Western Reserve University School of Medicine, Cleveland, O.*).

The results of this study confirm reports of previous authors that the Evans blue dye (T-1824) and the I^{131}-tagged albumin procedures for measuring plasma volume give comparable results. The radioisotope procedure is preferred because of the ease of handling for injection and blood determination by scintillation

counting. It also can be used repeatedly in the same patient without the complications inherent in the repeated injections of Evans blue dye.

An attempt to relate plasma volume directly to surface area, height, or weight by a simple ratio is mathematically incorrect unless the line of regression constructed from this relationship passes through the origin. The data presented here and that of others show a linear relationship which passes above the origin, indicating different ratios for different body builds. The smallest standard deviation from the line of regression occurred when plasma volume was plotted against weight or surface area. The slope for the line of regression for Evans blue dye related to surface area was: plasma volume = 1770 ml. × surface area M_2 − 460 ml. For body weight, the slope was: plasma volume = 668 ml. + 30 ml. × weight in kilograms.—*J. Lab. Clin. Med.* **45,** 841 (1955). (G. J. D.)

A method for the determination of glutamine in cerebrospinal fluid and the results in hepatic coma. T. P. Whitehead and S. R. F. Whittaker (*Warwick Hospital, England*).

To 1 ml. of spinal fluid in a 12 ml. test tube is added 0.2 ml. of 10% H_2SO_4. The tube is placed in a boiling water bath for exactly 10 minutes. After cooling in cold water, 0.3 ml. of 10% NaOH is added, followed by 5 ml. of water, 0.5 ml. of 2% gum ghatti solution and 2 ml. of Nessler's reagent. The color is read immediately, using a blue-green filter. A standard solution of glutamine is similarly treated. Urea in concentrations of 1% up to 400 mg./100 ml. is partially hydrolyzed under these conditions, and therefore the urea concentration of the spinal fluid is also determined and a correction made. Urea concentration in milligrams/100 ml. $\times \frac{5}{100}$ = milligrams of "glutamine." The glutamine content was also investigated using the one-dimensional, circular paper chromatographic technic of Giri and Rao [*Nature, London* **169,** 923 (1952)].

The normal range of glutamine in spinal fluid was found to be 6 to 14 mg./100 ml. In 9 patients with cirrhosis of the liver, the range was 16–31 mg., and in 7 cases of hepatic coma the values were from 30–54 mg. In a control series of patients in coma other than hepatic, the glutamine level was not elevated, with the exception of the fluid from 1 patient suffering from hemophilus meningitis.

The authors suggest that this simple and rapid procedure for the determination of glutamine is useful in the differential diagnosis of hepatic coma.—*J. Clin. Pathol.* **8,** 81 (1955). (E. V.)

Direct determination of uric acid using uricase. T. V. Feichtmeir and H. T. Wren (*Veterans Administration Hospital and University of California School of Medicine, San Francisco, Calif.*).

A method is described for the determination of uric acid in serum and in urine by a uricase procedure which is reported to obviate many of the technical difficulties of previous methods. To each of 2 tubes are added 0.5 ml. of serum, 0.5 ml.

of ⅔M glycine buffer and 4.0 ml. of water. To one of the tubes (unknown) is added 1.0 ml. of uricase. The other tube serves as a blank. Both tubes are incubated 2 hours at 45°. Four milliliters of 10% trichloroacetic acid and 1 ml. of uricase that has stood for 20 minutes are added to the blank tube and the contents are mixed by inversion. After standing for 20 minutes, both tubes are centrifuged at 3000 rpm for 15 minutes. The supernatants are transferred to clean tubes and recentrifuged to obtain crystal-clear solutions. The cuvet containing the unknown is set at zero optical density and the optical density of the blank tube is read in a Beckman Model DU spectrophotometer at 293 mμ. This reading gives the decrease in optical density caused by the action of uricase. Standard solutions of uric acid are prepared and run in the same way. The decrease in optical density of the unknown divided by the decrease in optical density of the standard, times the concentration of the standard, gives the concentration of the unknown.

The following reagents are required:

1. *Uric acid stock standard:* 0.2 Gm. recrystallized uric acid and 0.15 Gm. lithium carbonate dissolved in 30 ml. distilled water warmed to 60°. Shake until dissolved. Dilute to 200 ml. with water. Add a few drops of chloroform as a preservative. Stable 2 weeks at 4°. Concentration, 1 mg./ml.

2. *2/3M glycine buffer, pH 9.35:* 50 Gm. glycine; 400 ml. distilled water; 220 ml. of 1N NaOH. Adjust *p*H. Dilute to 1 L. and preserve with a few drops of chloroform.

3. 10% trichloroacetic acid.

4. 0.5 mg./ml. Worthington uricase (Worthington Biochemical Sales Co., Freehold, N. J.) in M/15 glycine buffer.—*Am. J. Clin. Pathol.* **25,** 833 (1955). (H. E. T.)

The gastric secretory response to histalog: One-hour basal and histalog secretion in normal persons and in patients with duodenal ulcer and gastric ulcer. J. B. Kirsner and H. Ford (*University of Chicago, Chicago, Ill.*).

Histalog (Eli Lilly Co., Indianapolis, Ind.) (3-beta aminoethylpyrazole), an analog of histamine, stimulates gastric secretion with less tendency to side effects than histamine. Histalog is administered subcutaneously in doses of 0.5 mg./Kg. of body weight. Outputs of HCl are larger than after histamine.—*J. Lab. Clin. Med.* **46,** 307 (1955). (G. J. D.)